ISSN 2095-8137 CN 53-1229/Q
Volume 38 Issue 1
18 January 2017

ZR

Zoological Research

KUNMING INSTITUTE OF ZOOLOGY, CAS
CHINA ZOOLOGICAL SOCIETY
Science Press
CODEN: DOYADI
www.zoores.ac.cn

ZOOLOGICAL RESEARCH

Volume 38, Issue 1 18 January 2017

CONTENTS

Editorial

New Year Address of *Zoological Research* ..... (1)

Obituary

In Memory of Academician Er-Mi Zhao (1930-2016) ..... (2)

Research highlight

Engrafted newborn neurons could functionally integrate into the host neuronal network .....

..... *Zheng-Bo Wang, Dong-Dong Qin, Xin-Tian Hu* (5)

Reviews

Consequences of early adverse rearing experience (EARE) on development: insights from non-human primate studies ..... *Bo Zhang* (7)

Does mRNA structure contain genetic information for regulating co-translational protein folding?

..... *Jian-Rong Yang* (36)

Reports

Expression of pIgR in the tracheal mucosa of SHIV/SIV-infected rhesus macaques .....

..... *Dong Li, Feng-Jie Wang, Lei Yu, Wen-Rong Yao, Yan-Fang Cui, Gui-Bo Yang* (44)

Nest survival rate of Reeves's pheasant (*Syrmaticus reevesii*) based on artificial nest experiments .....

..... *Xu Luo, Yu-Ze Zhao, Jing Ma, Jian-Qiang Li, Ji-Liang Xu* (49)

Cover image: *Macaca mulatta*. Photo by Xiao-Feng Ma **Cover design:** Lin Lei

New Year Address of *Zoological Research*

The year 2016 was a wonderful and important one for *Zoological Research* (*ZR*). In recognition of its impressive progress and great potential to develop into a leading journal in the field, *ZR* is now supported by the "Project for Enhancing International Impact of China STM Journals" (PIIJ) (Class B) (2016–2018). Having wide coverage, PIIJ is the most influential project in China for supporting and promoting the development of English language academic journal publications. In addition, *ZR* has achieved the "International Impact Academic Journal of China" title five years in a row.

The success of *ZR* depends on its unique niche in the field and the joint effort of its strong editorial team. Emphasizing the scope of *ZR*, namely, "Primates and Animal Models", "Conservation & Utilization of Animal Resources", and "Animal Diversity and Evolution", two special issues were released in 2016: "*The Amphibian and Reptile Biodiversity of Qinghai-Tibetan Plateau*" and "*CRISPR/Cas9, Model Animals and Human Diseases*". Moreover, to promote communication and collaboration among peer researchers, *ZR* is hosting the "2017 Frontiers in Zoology Symposium" in February 2017.

To further strengthen the editorial board and widen its scope of expertise, *ZR* continues to recruit local and overseas scientists to serve on its board. In 2016, *ZR* engaged four renowned scientists with varied expertise to join the vibrant *ZR* editorial team. These new board members include: Dr. Wai-Yee Chan, Chair Professor and Director, School of Biomedical Sciences, Chinese Hong Kong University, Hong Kong SAR, China; Dr. LeAnn Blomberg, Beltsville Agricultural Research Center (BARC), United States Department of Agriculture, USA; Dr. Hua-Xin (Larry) Liao, Professor, College of Life Science and Technology, Jinan University, China, and Professor, Duke University, USA; and Dr. Meng-Ji Lu, Professor, Institute of Virology, University of Duisburg-Essen, Germany. In addition, Dr. Guojie Zhang, a well-known expert on animal genomics who holds a position at the China National GeneBank, BGI-Shenzhen, and the Department of Biology, University of Copenhagen, as well as a guest professorship at the Kunming Institute of Zoology, Chinese Academy of Sciences, will also join the editorial board. With these new members, the editorial board of *ZR* now includes 48 distinguished scientists with expertise across multiple disciplines.

To further its scientific reach, *ZR* has continued to promote and market the journal during this past year. A new and more interactive homepage was launched and, as an open access journal, full-text articles can be downloaded via the homepage, PubMed Central, and SciEngine. To expand our readership, abstracts of published articles are also available online in Chinese. Information regarding the new initiatives and measures of *ZR* will continue to be shared with our readers through our official WeChat account (ZoolRes). In addition, *ZR* has now been indexed by Scopus and other related databases, and has a CitesScore value of 0.48 and ranks 786 among 1 549 journals in the subject "Medicine".

ZR will continue to publish innovative zoological studies and advance knowledge in scientific research. With the proud tradition of attracting outstanding scholars with diverse backgrounds and expertise to write for the journal, as well as the hard work and dedication of our editors and editorial board members, *ZR* has become a representative international scientific journal in animal studies. With a growing number of international submissions and article citations, as well as an increasing impact factor, we believe that *ZR* will confidently march into its thirty-seventh year and accomplish its goal of becoming an iconic journal in animal research. *ZR* could not have achieved what it has without the support of you, our readers. It is a journal that belongs to all members of the animal research community, and we look forward to continuing to work with you all to nurture and carry *ZR* to the next stage in 2017.

Sincerely

Wai-Yee Chan, Executive Editor-in-Chief
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The Chinese University of Hong Kong, China

Yong-Gang Yao, Editor-in-Chief
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In Memory of Academician Er-Mi Zhao (1930-2016)

Professor Er-Mi Zhao, Herpetologist, 1930-2016

Academician Er-Mi Zhao, Professor of the Chengdu Institute of Biology, Chinese Academy of Sciences, and Sichuan University, passed away on 24 December, 2016, to the great loss of both Chinese and world herpetology. As one of the most internationally renowned Chinese herpetologists, Academician Zhao represented a remarkable era of Chinese herpetology and made a significant contribution to global research, both academically and spiritually. He played a significant role in our understanding of the amphibians and reptiles of Tibet, herpetofauna of the Hengduan Mountains, taxonomy of Chinese snakes, and biogeography of the East Asian islands. His distinguished studies laid a solid and substantial foundation for research by future generations of young herpetologists, and had an extensive and profound impact on the development of the field.

As a prestigious scientist, Academician Zhao won the recognition and respect of his international peers and assured his place on the worldwide stage based on his remarkable academic achievements. “China should be so proud to have you representing her in herpetology research”, Roger Conant, a senior American scientist and *Agkistrodon* expert, wrote to Academician Zhao in 1989. In 1993, *Herpetology of China* (Zhao & Adler, 1993), the first book on the herpetofauna of China, which systematically described the 661 known species at the time, was co-authored by Academician Zhao and American scholar Prof. Kraig Adler. This great work took nearly half a century of meticulous investigation and ten years of compilation to achieve. The book was highly praised by Academician Ilya Darevsky of the Russian Academy of Sciences and Academician David Wake of the American Academy of Sciences, who claimed it to be “a milestone of herpetology literature” and “an epoch-making book, [which] inaugurates a new era in exploring the fauna of amphibians and reptiles of this vast land”.

As a diligent researcher, Academician Zhao devoted himself to herpetology research for 60 years. During his academic career, he published more than 140 articles, 43 books, including *Fauna Sinica* (Zhao et al., 1998), *Snakes of China* (Zhao, 2006), *Atlas of Chinese Snake*s (Hu et al., 1980), *Amphibian Zoogeographic Division of China* (Zhao, 1995), and *Herpetology of China* (Zhao & Adler, 1993) in five translations, and established four periodicals. Many of his works were compulsory study for herpetologists and students in China, America, England, and various other countries.

For herpetological classification and zoogeographic regionalization, Academician Zhao discovered a new record family of snake, *Cylindrophis ruffus* (Zhao & Adler, 1989), and 17 new record species of amphibians and reptiles in China. In addition, two new genera of amphibians, *Satobius* (*Hynobius retardatus*) (Adler & Zhao 1990) and *Liua* (*Liua shihi*) (Zhao & Hu, 1983), and 41 new species (or subspecies) were described and named by him, including *Japalura szechwanensis* (Hu & Zhao 1966), *Achalinus meiguensis* (Hu & Zhao, 1966), *Natrix optata* (Hu & Zhao, 1966). *Ranodon tsinpaensis* (Hu et al., 1966), *Megophrys nankiangensis* (Hu et al., 1966), *Hyla tsinlingensis* (Hu et al., 1966), *Rana kuangwuensis* (Hu et al., 1966), *Microhyla mixtura* (Hu et al., 1966), *Leiolopisma tsinlingensis* (Hu et al., 1966), *Xenopeltis hainanensis* (Hu et al., 1975), *Dinodon rosozonatum* (Hu et al., 1975), *Achalinus hainanus* (Hu et al., 1975), *Trimeresurus medoensis* (Zhao & Jiang, 1977), *Ovophis monticola zayuensis* (Zhao & Jiang,

DOI: 10.13918/j.issn.2095-8137.2017.006

1977), *Plagiopholis unipostocularis* (Zhao et al., 1978), *Opisthotropis guangxiensis* (Zhao et al., 1978), *Agkistrodon shedaoensis* (Zhao, 1979a), *Trimeresurus xiangchengensis* (Zhao, 1979b), *Phrynocephalus vlangalii hongyuanensis* (Jiang et al., 1980), *Oligodon multizonatum* (Zhao & Jiang, 1981), *Macropisthodon rudis multiprefrontalis* (Zhao & Jiang, 1981), *Scincella huanrenensis* (Zhao & Huang, 1982), *Scutiger ruginosus* (Zhao & Jiang, 1982), *Rhabdophis nuchalis pentasupralabialis* (Jiang & Zhao, 1983), *Platymantis reticulatus* (Zhao & Li, 1984a), *Staurois liangshanensis* (Wu & Zhao, 1984), *Calotes medogensis* (Zhao & Li, 1984b), *Tenuidactylus medogensis* (Zhao & Li, 1987), *Rana tenggerensis* (Zhao et al., 1988), *Eumeces liui* (Hikida & Zhao, 1989), *Trimeresurus mangshanensis* (Zhao & Chen, 1990), *Cuora zhoui* (Zhao et al, 1990), *Amphiesma metusia* (Inger et al, 1990), *Oreolalax multipunctatus* (Wu et al., 1993), *Rana robertingeri* (Wu & Zhao, 1995), *Rhabdophis adleri* (Zhao, 1997), *Laudakia wui* (Zhao, 1998a), *Laudakia papenfussi* (Zhao, 1998b), *Rana zhengi* (Zhao, 1999), *Opisthotropis cheni* (Zhao, 1999), *Rhacophorus hainanus* (Zhao et al., 2005), and *Gloydius lijianlii* (Jiang & Zhao, 2009), as well as the world-renowned Shedao Island pit viper (*Gloydius shedaoensis*) (Zhao, 1979a), Medog green pit viper (*Viridovipera medoensis*) (Zhao & Jiang, 1977), and Mt. Mang pit viper (*Protobothrops mangshanensis*) (Zhao & Chen, 1990). He also added eight new species for Tibet and 10 newly recorded species in China, and was the first to report on the king cobra in Medog (Zhao & Li, 1983) and expand its distribution northwards (Zhao et al., 1986).

Academician Zhao brought new insight and perspective on the geographical division of amphibians and reptiles in China, the amphibian and reptile fauna in the Hengduan Mountain area, the classification of snakes distributed in China, and the zoological geography of the East Asian Islands. He zoned the southern slopes of the Himalayas as a new sub-southwest region of the Indo-Chinese sub-region in the oriental realm according to the actual distribution of species in Tibet, and first proposed a new “South of the Himalayan Sub-region” based on the distribution of reptiles (Zhao et al., 1986). In addition, Academician Zhao also proposed new distribution patterns of amphibians in extra-tropical East Asia and the formation of amphibians and reptiles in the Western Pacific island chain (Zhao, 1989).

Professor Zhao also did outstanding work in scientific practice. He developed measures for the prevention and management of venomous snakes in the grasslands in western Xinjiang, and advanced the concept of medical geography of venomous snake bites in China (Qian et al., 1991) and Yunnan snake medicine to guide the practice of snake bite control. Collaborating with colleges and universities in Hainan, Professor Zhao recruited Masters and Doctoral aquaculture students to cultivate professional personnel for python breeding. He actively cooperated with Hainan python farms by providing systematic scientific knowledge.

Academician Zhao was not only a devoted scholar, but also played active roles in helping young researchers and in disseminating scientific knowledge. He served on the Executive Committee of the International Amphibian and Reptilian Society in 1983, and as the Chairman of Chinese Herpetologists in the IUCN (1991-2001). During his tenure as an academic advisor on the Editorial Board of *Zoological Research* from 1997 to 2014, he fulfilled his duty faithfully and diligently, and provided vital and constructive promotion of the journal. *Zoological Research* is extremely grateful for his exceptional contribution and dedication, and it is with deep regret and sorrow we note his passing. His deep passion, persistence, optimism, and strict scientific attitude in chasing the truth showed his invaluable spiritual wealth, and is an inspiration to every one of us.

Editor’s note: The facts and material stated here in regards to Academician Zhao’s academic achievements were provided by his students, Dr. Jia-Tang Li (Chengdu Institute of Biology, CAS), Dr. Jing Che (Kunming Institute of Zoology, CAS), Dr. Song Huang (Huangshan University, China), Peng Guo (Yibin University, China) and Dr. Yue-Zhao Wang (Chengdu Institute of Biology, CAS). This essay was compiled by *Zoological Research*.

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Engrafted newborn neurons could functionally integrate into the host neuronal network

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The limited capability to regenerate new neurons following injuries of the central neural system (CNS) still remains a major challenge for basic and clinical neuroscience. Neural stem cells (NSCs) could nearly have the potential to differentiate into all kinds of neural cells *in vitro*. Previous studies verified that exogenous transplanted NSCs are capable of differentiating into neurons and projecting onto the host neurons in the rat brain (Tabar et al., 2005; Dong JR et al., 2012), which could lead to behavioral recovery from neuronal damages such as spinal cord injury (McDonald et al., 1999), Parkinson's disease (Gonzalez et al., 2015; Kim et al., 2002; Lindvall, 2001), and stroke (Zhang et al., 2016). In January, 2013, the Food and Drug Administration (FDA) of United States has given Neuralstem clearance to commence its human spinal stem cell transplantation in chronic spinal cord injury (Orelli, 2013). In addition, this company is evaluating the safety of stem cell transplantation for the treatment of major depressive disorder (MDD), chronic traumatic encephalopathy (CTE), Alzheimer's disease, and post-traumatic stress disorder (PTSD) (Neuralstem Inc., 2013). However, the mechanism underlying this treatment is controversial. It is still unknown whether the secretion of trophic factors by the transplanted NSCs slowed or prevented the deterioration of the degenerating neurons (Breunig et al., 2011; Xiao et al., 2015; Zuo et al., 2015), or the new neurons differentiated from the transplanted NSCs substituted the injured or lost neurons by functionally integrating into the host neural circuitry (Englund et al., 2002; Weick et al., 2011). To illustrate this question and find out how likely the injured neurons were substituted with new neurons, the functional examination is preferentially to be carried out on an intact animal model (or awake animal). Generally, stem cell clusters transplanted into the brain parenchyma will migrate as single cells towards diverse brain regions. In view of this, approaches that could lock the engrafted cell mass in the specific site were required (Yang SC et al., 2011). Herein, a new technique was developed where a small 'hole' was created in the inferior colliculus (IC) of rhesus monkeys to lock the transplanted NSCs in situ and investigate their integration into the host auditory neural network. The results showed that a substantial portion of transplanted cells differentiated into mature neurons and formed synaptic input/output connections with the host neurons. Under awake condition, c-fos expression in newborn neurons was found to be significantly increased after acoustic stimulation and multichannel recordings indicated IC specific tuning activities in response to auditory stimulation were also recorded in newborn neurons as the reported IC neurons (Zwiers et al., 2004). These results suggest that the transplanted cells have functionally integrated into the host neural network in the awake monkey brain and provided a strong foundation for the future stem cell treatment of the CNS injuries (Wei et al., 2016).

This is the first time evaluating the neurons differentiated from the transplanted NSCs in awake animal, which was further confirmed by Mark Hübener and his group working at the Max Planck Institute of Neurobiology (Falkner et al., 2016). They also found that the transplanted neural stem cells could differentiate into local neuronal phenotype, target projection and functionally integrate with the host neurons. Both of the findings revealed that engrafted stem cells could differentiate into mature neurons, form synaptic with the host neurons, successfully substitute the injured or lost neurons and functionally integrate with the host neuronal network, which might be necessary for the normal brain function of awake animal.

The current findings provide impetus for stem-cell therapy, but if you want to fully assess the ability of transplanted stem cells to differentiate and replace lost neurons in a damaged brain, further sophisticated experimentation should be conducted. For example, the high-throughput dual-color precision imaging (Gong et al., 2016) or transparent intact brain technique (Ke et al., 2013; Susaki et al., 2014; Yang et al., 2014)

Received: 20 December 2016; Accepted: 10 Janurary 2017

Foundation items: This research was supported by National Program on Key Basic Research Project (973 Programs 2015CB755605) and National Natural Science Foundation of China (81471312)

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DOI:10.13918/j.issn.2095-8137.2017.005

can be used to reconstruct the engrafted neurons' projections with the host, from which we can overview the newborn neurons and their integration with the host from a systems perspective. All in all, these findings provide encouragement that stem cell replacement therapy will become a reality in the near future.

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ZOOLOGICAL RESEARCH

Consequences of early adverse rearing experience (EARE) on development: insights from non-human primate studies

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ABSTRACT

Early rearing experiences are important in one's whole life, whereas early adverse rearing experience (EARE) is usually related to various physical and mental disorders in later life. Although there were many studies on human and animals, regarding the effect of EARE on brain development, neuroendocrine systems, as well as the consequential mental disorders and behavioral abnormalities, the underlying mechanisms remain unclear. Due to the close genetic relationship and similarity in social organizations with humans, non-human primate (NHP) studies were performed for over 60 years. Various EARE models were developed to disrupt the early normal interactions between infants and mothers or peers. Those studies provided important insights of EARE induced effects on the physiological and behavioral systems of NHPs across life span, such as social behaviors (including disturbance behavior, social deficiency, sexual behavior, etc), learning and memory ability, brain structural and functional developments (including influences on neurons and glia cells, neuroendocrine systems, e.g., hypothalamic-pituitary-adrenal (HPA) axis, etc). In this review, the effects of EARE and the underlying epigenetic mechanisms were comprehensively summarized and the possibility of rehabilitation was discussed.

Keywords: Early adverse rearing experience; Non-human primates

INTRODUCTION

One of factors affecting life-long health of humans is the stability of early childhood, especially children's relationship with their mothers. John Bowlby's attachment theory suggests that individual's social relationship throughout life is influenced by the initial attachment with the mother (Bowlby, 1969). Attachment theory is a psychological, evolutionary and ethological theory concerning relationships among humans. Within the theory, attachment means an affectional bond or tie between an individual and an attachment figure (usually a caregiver). The core is that a child needs to build relationship with at least one primary caregiver to develop normal social and emotional behaviors. In many orphans, the lack of normal attachment to parents would cause behavioral and physical problems in childhood and possibly continuing throughout adult life (McEwen, 2003). Adults with adverse experience were more vulnerable to physical, psychosocial and mental disorders (Maughan & McCarthy, 1997; Pirkola et al., 2005). In human, early adverse rearing experience (EARE) usually refers to child abuse, which is a worldwide problem and is defined as neglect or physical, sexual or emotional mistreatment or abuse of children (Newton & Vandeven, 2009, 2010). Although human based studies revealed compelling associations between EARE and psychological outcomes, both retrospective and prospective studies showed their limits, e.g., inaccurate self-report due to biased or even false memory, failure in controlling accompanying environmental and genetic factors. Therefore, the long-term effects of EARE on subjects were usually not the direct consequences, but were inevitably intervened or masked by uncontrollable factors. However, experimental animals can be raised in laboratory environments, therefore allow researchers to carry out randomized prospective longitudinal studies, e.g., rigorously control or systematically manipulate early experiences throughout the entire period of investigation.

Received: 01 December 2016; Accepted: 30 December 2016

Foundation items: This research was supported by Hainan special fund project for science and technology (KJHZ2015-20)

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DOI:10.13918/j.issn.2095-8137.2017.002

Rodents are easy to manipulate genetically, and the related studies indicate EARE as a developmental risk factor with profound, long-term effects on later life (Meaney, 2001; Pryce et al., 2005b; Sánchez et al., 2001). Whereas the high similarities of NHPs with humans make it irreplaceable in investigating the effects of EARE on physiological and behavioral development, e.g., NHPs and chimpanzees in specific, share over 90% and 98.8% genomes with human beings, respectively (Lovejoy, 1981). High similarities were found in both biological (Azmitia & Gannon, 1986; Uylings & van Eden, 1991) and socio-ecological aspects, e.g., social organizations and clear dominance hierarchies (Bailey & Aunger, 1990; DeVore, 1990; Wright, 1990). The phenomenon that in NHPs, 2%-10% of infants were physically abused or neglected by their mothers in group-living conditions, allow the possible screening of natural child abuse models (Maestripieri & Carroll, 1998; Maestripieri et al., 1997). Moreover, like humans NHPs has prolonged postnatal period of maturation during which mother–infant relationship and neural system development can be influenced by environment and early life experience (Levine & Wiener, 1988; Suomi, 2005).

Harlow (Harlow & Harlow, 1965) introduced the concept of affectional systems to characterize the relationships in the social groups of primates, and five distinct affectional systems were described, including the infant-mother affectional system, the maternal affectional system, the age-mate/peer affectional system, the heterosexual affectional system and the paternal affectional system. The infant-mother and the maternal affectional systems in Harlow's affectional systems are similar to Bowlby's concept of mother-infant attachment theory in humans. In normal living group, most monkey infants virtually spend all of their initial days or weeks of life clinging with their biological mothers, ventral to ventral, during which, specific and strong attachment bonds are built. When about 2-month old, infants begin to explore the physical and social environment, spending increasing amount of time participating social interactions, especially playing with peers. From 6-month of age until puberty, playing with peers becomes the major social activity (Hinde & Spencer-Booth, 1967; Suomi, 1997, 2005). In fact, the infant and juvenile monkeys always maintain a close social relationship with their mothers, while the mother plays the role of protector especially under stressful situations, and mentor in teaching developing appropriate social behaviors. Accordingly, the studies regarding EARE usually involve disruption of the normal infant-mother relationships, by maternal deprivation of newborns, maternal separation or induced stress on older infants and juvenile monkeys. Although some epidemiological studies in humans suggest possible direct relationships between EARE and abnormal behaviors in later life, no solid evidence was raised to prove the precise impact of childhood adversities on psychiatric disorders (Benjet, 2010; Bick & Nelson, 2016; Gershon et al., 2013; Kessler et al., 1997; Kessler & Wang, 2008; Klein et al., 2013; Sheridan et al., 2010). The over 60 years NHP studies shed lights on the understanding of the influences of EARE on physiological and behavioral development, including social behaviors (e.g., disturbance behavior, social deficiency, sexual behavior, etc), learning and memory ability, brain structural and functional development (e.g., development of neurons and glia cells, neuroendocrine dysregulation, etc). In this review, the previous findings on EARE were systematically summarized, and the underlying epigenetic mechanisms and the potential methods of rehabilitation were thoroughly discussed.

EARE MODELS IN NON-HUMAN PRIMATES

Controlled rearing conditions in standard laboratory settings are designed to simulate natural environments. The infants are reared by their mothers and live in a group consisted of other infants, juveniles and adults, allowing infants to be exposed to complex social interactions. In abnormal rearing conditions, the mother deprivation method is applied. The newborn is taken away from their mothers at birth and is reared in incubators with regular medical attention and laboratory nursery. A period of time (usually 1-month) later, when able to feed themselves, infants are moved to other rearing conditions depending on aims of research, e.g., be reared alone in social isolation condition, with nursery/peers of the same age in nursery/peer rearing condition, with a surrogate in surrogate mother/foster rearing condition, etc. They could also be separated from mothers at later time for once (temporary maternal separations) or several times (repetitive maternal separations); or even though staying with their mother all the time, but still suffer from EARE (maternal neglect).

Social isolation

Social isolation (including total and partial social isolation) is initially described in early 1960s by Harlow and his colleagues, and has been used ever since to raise monkeys in simulating social behavior deficits in humans (Table 1). In total isolation, the infant is reared in a cage alone without any auditory, visual, olfactory and tactile contact with conspecifics, including mothers, peers and other monkeys (Baysinger et al., 1972; Harlow & Harlow, 1962; Harlow et al., 1964, 1965). In partial social isolation, although infants are separately caged from their mothers, peers, and social groups, they have auditory, visual, and olfactory but not tactile contact with their conspecifics (Cross & Harlow, 1965; Mason & Sponholz, 1963; Struble & Riesen, 1978; Suomi et al., 1971). These early studies by Harlow and his colleagues, especially their extreme manipulations, including total isolation, "pit of despair" and "rape rack" devices, were controversial and were most likely forbidden to perform due to ethical issues. In 1950s, many researchers assumed that the only necessity of mother was supplying food to infants, whereas excessive intimacy between mother and infant would hinder the growth of infant, or even induce over dependence in adulthood. Harlow disagreed with the viewpoints; performed a series of isolation studies on primates to prove that to acquire necessary social skills, to obtain both physically and psychologically healthy development, infants need mothers' affection, as well as normal social interaction and emotional relationship with peers. However, their intention was to prove the essential role of mother's love to infants, in the

Table 1 Early adverse rearing experience (EARE) methods

Paradigms		Description			References
Social Isolation	Total	Infants are reared in a cage alone at birth, no any auditory, visual, olfactory and tactile contact with conspecifics is allowed			Baysinger et al., 1972; Harlow et al., 1965; Harlow & Harlow, 1962; Harlow et al., 1964
	Partial	Infants are separately caged at birth, reared with auditory, visual, and olfactory, but not tactile contact with conspecifics			Cross & Harlow, 1965; Mason & Sponholz, 1963; Struble & Riesen, 1978; Suomi et al., 1971
Maternal separation	Permanent	Peer-rearing	Continuous	Infants are reared by pairs throughout development	Chamove et al., 1973; Erwin et al., 1973; Sackett, 1967; Worlein & Sackett, 1997
			Intermittent	Peers are allowed to contact with each other for a limited period of time and then infants are housed singly during the rest of the time	Rommeck et al., 2009b
			Rotational	Infants are continuously housed with different peers	Novak & Sackett, 1997; Rommeck et al., 2009b
		Surrogate mothers rearing (SMR)	Inanimate objects are placed into the cage as an artificial surrogate mother		Capitanio & Mason, 2000; Dettmer et al., 2008; Schneider & Suomi, 1992; Suomi, 1973
		Surrogate-peer rearing (SPR)	Combination of SMR and PR		Bastian et al., 2003; Lutz et al., 2007; Meyer et al., 1975
	Temporary	One time	Infants are taken away from their mothers at later stages of life for a period of time, followed by mother-infant reunion		Hinde & Mcginnis, 1977; Hinde et al., 1966; Kaufman & Rosenblum, 1967; Seay et al., 1962; Spencer-Booth & Hinde, 1971
		Repetitive	Repeatedly separating infants from their natal group for relatively short periods of time, followed by repeated reunions		Clarke et al., 1998; Dettling et al., 2002a, b; Levine & Mody, 2003; Sánchez et al., 2005; Suomi et al., 1983
Maternal neglect			Infant-mother was confronted with various foraging conditions to induce different levels of stress in the mother		Andrews & Rosenblum, 1991; Coplan et al., 1996; Rosenblum & Andrews, 1994; Rosenblum & Paully, 1984

form of her availability all the time, her physical touching, caring and protection, which was an obvious fact to us today without any necessity to prove.

However, although isolation models are important in highlighting the devastating consequences of maternal deprivation, the extreme manipulations could induce severe cognitive and emotional deficits, or even self-injurious behaviors, which are very difficult to remediate in primates. Therefore, less severe rearing conditions were developed afterwards at least partially due to ethical considerations.

Maternal separations

Peer-rearing (PR) (or nursery rearing, NR) (including continuous pair rearing, intermittent and rotational peer rearing) is another widely used rearing condition, in which infants were reared together with peers of the same age (Chamove et al., 1973; Erwin et al., 1973; Sackett, 1967; Worlein & Sackett, 1997) (Table 1). In continuous pair rearing condition, infants are usually reared by pairs throughout development (Chamove et al., 1973; Fekete et al., 2000; Hotchkiss & Paule, 2003; Novak & Sackett, 1997). Intermittent peer rearing allow peers to contact with each other for a limited period of time, and then infants are housed singly during the rest of the time (Rommeck et al., 2009b). Within the rotational peer rearing condition, infants are continuously peer housed with different infant partners (Novak & Sackett, 1997; Rommeck et al., 2009b). Previous study showed that continuous rotational pairing induces a behavioral profile quite similar with that of mother rearing in socially complex environment (Rommeck et al., 2011). Compared with social isolation, PR is less severe and thus more widely used in recent NHP EARE studies. Surrogate mothers rearing (SMR) is another early rearing method, in which inanimate object is placed into the cage as an artificial surrogate mother (Capitanio & Mason, 2000; Dettmer et al., 2008; Eastman & Mason, 1975; Harlow, 1958; Harlow & Zimmermann, 1959; Hennessy & Kaplan, 1982; Kaplan, 1974; Mason & Berkson, 1975; Roy et al., 1978; Schneider & Suomi, 1992; Suomi, 1973). Infants could quickly develop attachment with surrogate mothers, and some studies indicated that the infants usually preferred cloth surrogate mothers than wired ones (Harlow, 1958; Harlow & Zimmermann, 1959). Previous study reported that surrogate mothers could affect the behaviors of infants, and different characters of surrogate mothers such as mobility and orientation had different influences (Dettmer et al., 2008). Surrogate-peer rearing (SPR) method is a combination of SMR and PR, in which the infants are reared with inanimate surrogate mothers (SMR condition) during the initial several months of life, and then are allowed to have peer interactions for a limited period of time (PR condition) (Bastian et al., 2003; Lutz et al., 2007; Meyer et al., 1975). Comparing with permanent removal of the mother, infants are not separated from their mothers right away at born in

temporary maternal separations, but after a period of time usually several hours, days or weeks, following by mother-infant reunion (Hinde et al., 1966; Hinde & McGinnis, 1977; Kaufman & Rosenblum, 1967; Seay et al., 1962; Spencer-Booth & Hinde, 1971). Temporary maternal separation usually contains a one-time separation although different time delay could be adopted. A modified version of one-time separation is repetitive mother-infant separation, in which infants are separated from and reunited with their natal group repeatedly for relatively short periods of time (Clarke et al., 1998; Dettling et al., 2002b; Levine & Mody, 2003; Sánchez et al., 2005; Suomi et al., 1983). The impact of these procedures appeared to be further intensified if the separations were unpredictable (Levine, 2000; Sánchez et al., 2005). Unlike social isolation, maternal separation adopted relatively mild manipulations, the presence of surrogate mothers and the opportunity of direct contact with mothers and peers added social complexity to the infants' living environment, therefore could avoid severe social and emotional deficits associated with mothers' absence.

Maternal neglect

Compared with isolation and maternal separation methods described above, maternal abuse and neglect during early life are more common in humans, therefore are more widely used on NHPs to study adult mood and anxiety disorders. In NHP maternal neglect models, in order to induce stress in the mother, infant mothers are confronted with various foraging conditions, such as variable/unpredictable foraging demand (VFD), consistently low foraging demand (LFD) and consistently high (but predictable) foraging demand (HFD). Mothers in LFD condition have easy access to food while those in high foraging demand have to work hard to get food (Andrews & Rosenblum, 1991; Coplan et al., 1996; Rosenblum & Andrews, 1994; Rosenblum & Paully, 1984). The advantage of this model is that even though infants are still in adverse situation, the severe adverse experience of mother and peer deprivation can be avoided. In addition, other rearing strategies are applied in this model, i.e., infants were reared by a female which was not their biological mother (Maestripieri, 2005; Novak & Suomi, 1991); infants were housed with non-reproductive female adults (Champoux et al., 1989b).

EARE EFFECTS

Although partial social isolation tends to induce less severe defects than total social isolation, the expression of behavior defects is similar. Isolated monkeys reared without exposure to companions during early life, especially the first 6 months, develop a pervasive pattern of abnormalities referred to as the isolation syndrome. Mason (Mason, 1968) summarized the syndrome under four headings: (1) abnormal posturing and movements, such as rocking; (2) motivational disturbances, such as excessive fearfulness or arousal; (3) poor integration of motor patterns, such as inadequate sexual behavior; (4) deficiencies in social communication, such as failure to withdraw after being threatened by an aggressing animal. In this section, the effects of EARE on social behaviors, learning and memory ability, brain structural and functional developments, including influences on neurons and glia cells, neuroendocrine dysregulation, especially stress related HPA axis will be reviewed.

Social behavior

Effects of EARE on social behaviors are detailed in Table 2.

Table 2 Effects of EARE on social behaviors

Behavior types	Behavior descriptions
Stereotypic behaviors	Whole-body stereotypes (e.g., rocking, pacing, bouncing, swing, and back-flipping)
	Self-directed stereotypes (e.g., saluting, digit-sucking, self-clutching, self-clasping, eye-poking, eye-covering and hair-pulling)
Self-directed behaviors	Self-manipulation, self-scratching, self-grasping, self-rubbing
	Self-injurious behavior (SIB)
Aggression	Less aggression during infancy and more aggression during later life
Affiliative behavior	Tend to show more affiliative behavior during infancy but less affiliative behavior during adulthood
Social and environmental exploration	Decreased social and environmental exploration
Social dominance	Tend to show low dominance rank
Sexual behaviors	Less and abnormal sexual behaviors
Others	Polyphagia and polydipsia

Disturbance behavior

Monkeys exposed to adverse early experience tended to show more disturbance behaviors, such as stereotypic and self-directed behaviors, motivational disturbances and social deficiency. The isolated monkeys appeared to show more disturbance behaviors, including crouching, clutching, rocking, pacing, flipping, hugging, clasping, thumb-sucking (Harlow & Harlow, 1962; Harlow & Suomi, 1971a; Mason & Sponholz, 1963; Mitchell, 1968; Suomi et al., 1971). Among these monkeys, some abnormal movements, such as rocking and self-grasping, could present very early in their lives, even at the first month (Baysinger et al., 1972). Additionally, some of these behaviors could turn into stereotypic behaviors, including repetitive movements or postures, as well as ritualized movements, and could be divided into whole-body stereotypes

(e.g., rocking, pacing, bouncing, swing, and back-flipping), self-directed stereotypes (e.g., saluting, digit-sucking, self-clutching, self-clasping, eye-poking, eye-covering and hair-pulling) and other idiosyncratic behaviors (e.g., teeth grinding, head tossing, or making noise by blowing air into the cheeks). It was reported that whole-body stereotypes were much more common than self-directed stereotypes (Lutz et al., 2003). Previous studies indicated that isolated monkeys showed more repetitive whole-body stereotypes (Mitchell, 1968), while PR monkeys showed more self-directed stereotypes (Lutz et al., 2003; Suomi et al., 1971).

EARE exposed monkeys tended to show more self-directed behaviors. Isolated monkeys showed self-manipulation, self-scratching, self-grasping, self-rubbing, and autoeroticism while in isolation (Baysinger et al., 1972), or showed remarkable increases in self-clasping soon after removal from isolation (Harlow et al., 1965; Suomi et al., 1974), or self-clutching after surrogate mother removing (Harlow & Zimmermann, 1959). PR reared infants and juvenile monkeys showed increased self-stimulation behaviors, including self-sucking, self-clinging, self-clasping and other self-directed behaviors (Champoux et al., 1991; Lutz et al., 2003; Suomi et al., 1971). Moreover, short-term stress by temporary physical restrictions could also induce significant increases in self-clasping and huddling behaviors when the infants returned to their home cages (Harlow & Suomi, 1971a). Those self-directed behaviors often turned into self-injurious behavior (SIB), with males showing a much higher level of vulnerability than females (Cross & Harlow, 1965; Gluck & Sackett, 1974; Lutz et al., 2003; Suomi et al., 1971), and PR monkeys usually much more vulnerable than MR monkeys (Rommeck et al., 2009a). Surrogate mothers appeared to provide a certain degree of contact acceptability, security and trust sufficient for isolated monkeys to suppress existing self-directed disturbance activity, and to initiate crude social interactions with other isolated monkeys (Harlow & Suomi, 1971b). However, Lutz et al. (Lutz et al., 2007) reported that SPR monkeys showed significantly more self-biting comparing to PR and MR reared animals, and it was suggested that surrogate rearing in combination with lower levels of social contact during play may be risk factors for the later development of self-biting behavior. Actually, self-directed behaviors were hypothesized to result from the redirection of normal social behaviors toward one's own body and were suggested to be symptoms of some mental diseases (Goosen, 1981; Mason & Berkson, 1975). These findings indicate that EARE exposed monkeys could be used as an ideal model of related human mental disorders from behavioral perspective.

Social deficiency

In natural environments, infants and juvenile monkeys are supposed to be more active in joining the social play with peers, but monkeys exposed to EARE show decreased social playing. Isolated monkeys showed less (Harlow et al., 1965; Mitchell, 1968), or even no contact playing at all (Harlow et al., 1965). Pair and peer reared infants (Chamove et al., 1973), VFD reared infants (Andrews & Rosenblum, 1991; Rosenblum & Paully, 1984), repeated parental deprivation infants (Dettling et al., 2002b; Levine & Mody, 2003) all showed less social playing compared with MR infants. Lack of sufficient social interaction led to the fact that EARE exposed monkeys could not successfully adapt to living in a large social group (Griffin & Harlow, 1966; Harlow & Harlow, 1962; Mason & Sponholz, 1963; Ruppenthal et al., 1991). Not only social interaction, studies also showed decreased environmental exploration in isolated monkeys (Griffin & Harlow, 1966; Mason & Sponholz, 1963; Mitchell, 1968), VFD and PR monkeys (Rosenblum & Paully, 1984; Ruppenthal et al., 1991). Another major index of exploratory behavior is locomotor activity, while some NHP studies showed less locomotion in isolated adults (Harlow & Suomi, 1971a; Mason & Sponholz, 1963; Mitchell, 1968) and PR infants (Feng et al., 2011), others found no differences in PR adults (Winslow et al., 2003), or even higher activity levels in PR infants during the first month after isolation (Champoux et al., 1991). Therefore, there was no agreed tendency of EARE influence on locomotor activity in monkeys, making it an invalid measure of exploratory behavior if used alone (Wright, 1983).

Another domain of EARE induced social deficiency is social dominance. In monkey society, social dominance is a complex phenomenon mediated by different mechanisms and various factors such as kinship, age, sex, and physical factors like body weight, appearance and health (Bernstein & Cooper, 1999; Bernstein & Mason, 1963; Morgan et al., 2000; Sprague, 1998; Takahashi, 2002). Kinship seemed to be the major factor in determining dominant rank at least until puberty (Koford, 1963; Koyama, 1967), but became weaker during the development (Bernstein & Williams, 1983). Both dominance formation and maintenance among males in a living group are usually achieved by aggressive behavior such as fighting, with the stronger and more aggressive subjects winning and thus becoming dominant. However, appropriate use of aggression is critical for both acquiring and maintaining social status, as overly aggressive monkeys may risk social ostracism from their conspecifics. Moreover, aggressive behavior was not indispensable to obtain and keep dominance status and dominance sustained without aggression was more stable than that formed on the basis of aggression (Fonberg, 1988).

Monkeys exposed to EARE tended to show less aggression during infancy (Chamove et al., 1973; Harlow et al., 1965), and more aggression during later life (Chamove et al., 1973; Mitchell, 1968; Suomi et al., 1974; Winslow et al., 2003). The aggressive monkeys exposed to EARE may repeatedly attack a helpless infant or attempt to attack a dominant male, while infant-directed aggression is abnormal adult-directed aggression is both abnormal and suicidal (Chamove et al., 1973; Mitchell, 1968; Suomi et al., 1974; Winslow et al., 2003). On the other hand, studies showed EARE exposed monkeys showed heightened fear in all age stages (Champoux et al., 1991; Dettling et al., 2002b; Levine & Mody, 2003; Mitchell, 1968). It seems that EARE makes monkeys more emotional in two opposite directions, both aggression and fear. In addition to aggression, affiliative behavior, such as grooming and proximity, is also important in establishing and maintaining alliances and reinforcing the dominance hierarchy. Affiliative behavior was suggested to be more positively related to dominance rank than kinship in Japanese monkeys (Singh et al., 1992). On the

contrary to aggression, EARE exposed monkeys showed more affiliative behavior during infancy (Chamove et al., 1973; Rosenblum & Paully, 1984; Ruppenthal et al., 1991), but less affiliative behavior during adulthood (Kraemer & McKinney, 1979; Rosenblum & Paully, 1984; Winslow et al., 2003). With more aggressive and less affiliative behavior which both contribute to acquiring and reinforcing social dominance, EARE exposed adult monkeys are supposed to have low social dominant rank in a living group, and studies indeed indicated that both isolated and PR adult monkeys showed low social dominance (Kraemer & McKinney, 1979; Mitchell, 1968; Ruppenthal et al., 1991).

Sexual behavior

Monkeys exposed to EARE demonstrated less or abnormal sexual behaviors (Chamove et al., 1973; Harlow et al., 1966; Harlow, 1962; Harlow et al., 1965; Mitchell, 1968). Abnormal sexual behaviors (abortive mount) is defined as any improperly oriented mount, accompanied by pelvic thrusting including standing-to-head, standing-to-side and ventral lie-on (Wallen et al., 1981). Males usually were not mount properly as they engaged in varied but misplaced heterosexual efforts, while females were not maintain the sexual present (stood quadripedally with the perineal area directed towards the recipient) or turned their bodies when mounted. Mount behavior includes no-foot-clasp mount and foot-clasp mount, which could be differentially affect by different EARE. Males with short access periods with peers (0.5 h) rarely or never foot-clasp-mounted peers, while those given 24 h access regularly foot-clasp-mounted peers (Wallen et al., 1981). Isosexually reared males showed less foot-clasp mounting and more presenting than heterosexual males, while conversely, isosexually reared females showed statistically more mounting and less presenting than heterosexual females (Goldfoot et al., 1984). Moreover, females exposed to EARE also showed abnormal maternal behaviors, in a way that those never experienced mother caring not only were unable to exhibit caring to their own offspring, but also far more likely to display inadequate, abusive or neglectful behavior toward their offspring (Bridges et al., 2008; Champoux et al., 1992; Harlow & Suomi, 1971b; Seay et al., 1964; Suomi, 1978; Suomi et al., 1974; Suomi & Ripp, 1983), consistent with human findings showing abusive behavior appeared to be transmitted across generations (Roustit et al., 2009).

Primate studies also showed other EARE induced behavioral effects besides listed above, including polyphagia and polydipsia in isolated adults (Miller et al., 1969), more vulnerable to excessive alcohol consumption (Fahlke et al., 2000; Higley et al., 1991) and elevated response to both aversive and rewarding stimuli (Nelson et al., 2009) in PR monkeys and abnormal sleep rhythmicity (Barrett et al., 2009; Boccia et al., 1989; Kaemingk & Reite, 1987; Reite et al., 1974; Reite & Short, 1978). An interesting research showed EARE significantly influenced the development of lateralisation, as PR monkeys demonstrated greater left-hand bias compared to MR reared monkeys (Bennett et al., 2008). Despite of EARE effects described above, recent research suggested that modern PR practices might not result in inevitable perturbations in aggressive, rank-related, sexual, and emotional behavior in rhesus monkeys (Bauer & Baker, 2016).

Learning and memory

Early primate studies showed EARE exposed adults performed adequately on simple discriminations or delayed-response (Gluck et al., 1973), but showed impairments in certain complex tasks such as those requiring engaging working memory with dynamic rules or delays or response inhibition (Beauchamp & Gluck, 1988; Beauchamp et al., 1991; Gluck et al., 1973; Gluck & Sackett, 1976; Sánchez et al., 1998). These results were obtained mostly from adult monkeys separated from their mothers at birth and reared in total isolation for 9-12 months. PR reared juvenile monkeys also showed cognitive deficits, they had more difficulty acquiring the delayed non-matching to sample (DNMS) task and were also impaired in object but not spatial reversal learning (Sánchez et al., 1998). Moreover, even brief social isolation impaired performance in a multiple video-task assessment in adult rhesus monkeys (Washburn & Rumbaugh, 1991) and impaired reversal learning and behavioral inhibition in adult marmosets (Pryce et al., 2004a, b). These results were consistent with the results of human studies, which showed the post institutionalized children (Bauer et al., 2009) and childhood exposed to neglect and abuse (Majer et al., 2010) were associated with impaired learning and memory during adulthood. Although those studies revealed EARE induced impairment of learning and memory ability in a task dependent way in adult monkeys, other primate studies indicate exposure to mild early life stress improves prefrontal dependent response inhibition in primates, suggesting its beneficial effect on cognitive control (Parker et al., 2005, 2012).

Brain structure and function

The first documentation of the effects of negative early experiences on monkey brain was provided by Martin et al. (1991), which showed significant alterations in the chemo architecture of the striatum 19-24 years after social deprivation. Additionally, Siegel et al. (1993) demonstrated that early social deprivation resulted in an increase in the amount of non-phosphorylated neurofilament protein in hippocampal dentate gyrus granule cells in rhesus monkeys. Further studies showed structure and function changes in many brain regions including amygdala, hippocampus, prefrontal cortex (PFC), anterior cingulate cortex (ACC), corpus callosum and cerebellum etc, both in humans and animals exposed to EARE (Andersen, 2015; Bick & Nelson, 2016; Gilmer & McKinney, 2003; Gorman et al., 2002; Hart & Rubia, 2012; Korosi et al., 2012; McEwen, 2003; Worlein, 2014)(Table 3).

Amygdala

Amygdala is a group of almond-shaped nuclei located deep within the medial temporal lobes of the brain in complex vertebrates. It was considered as the emotion center and responsible for emotion reactions like reward, fear and anxiety (Davis, 1992; Gallagher & Chiba, 1996; Ledoux, 2003; Phelps, 2006). Rodent studies showed acceleration of amygdala development in early weaning rodents (Kikusui & Mori, 2009;

Table 3 Effects of EARE on brain structure and function

	Outcomes		Human Studies	Primate Studies
Amygdala	Children	No significant volumes changes	De Bellis et al., 2001; De Brito et al., 2013; Hanson et al., 2010; Woon & Hedges, 2008	No significant volume changes (Howell et al., 2014);
		Larger volume and elevated response	Lupien et al., 2011; Mehta et al., 2009; Tottenham et al., 2010	Decreased SERT binding potential (Ichise et al., 2006);
		Decreased volume	Edmiston et al., 2011; Hanson et al., 2015; Luby et al., 2013	Differential expression of one gene GUCY1A3 (Sabatini et al., 2007)
	Adults	No significant volume changes	Bremner et al., 1997; Cohen et al., 2006	
		Larger volume	Evans et al., 2016; Lyons-Ruth et al., 2016	
		Elevated activity	Casement et al., 2014; Javanbakht et al., 2015; Kim et al., 2013	
Hippocampus	Children	Decreased volume	Edmiston et al., 2011; Hanson et al., 2015; Luby et al., 2013	No significant volume change (Law et al., 2009a, b; Sánchez et al., 1998; Spinelli et al., 2009)
		No significant volume change	Carrion et al., 2001; De Bellis et al., 2001; De Bellis et al., 1999; De Bellis et al., 2002; Mehta et al., 2009; Tottenham et al., 2010; Woon & Hedges, 2008	
	Adults	Decreased volume	Bremner et al., 1997; Cohen et al., 2006; Stein et al., 1997; Woon & Hedges, 2008	
Prefrontal cortex (PFC)	Children	No significant volume changes	De Bellis et al., 1999	Greater enlarged medial prefrontal cortex (mPFC) size (Spinelli et al., 2009)
		Decreased volume	De Bellis et al., 2002; Edmiston et al., 2011; Hanson et al., 2010; Morey et al., 2016; Thomaes et al., 2010	
		Larger volume	Carrion et al., 2009; Richert et al., 2006	
		Decreased volume	Tomoda et al., 2009; van Harmelen et al., 2010	
	Adults	Reduced activity	Casement et al., 2015; Kim et al., 2013; Romens et al., 2015; Schweizer et al., 2016	
		Increased response	Casement et al., 2014; Javanbakht et al., 2015; Jedd et al., 2015; Wang et al., 2016; White et al., 2015	

Ono et al., 2008). The limited amount of primate studies found no significant amygdala volume changes (Howell et al., 2014), but functional changes including decreased SERT binding potential (Ichise et al., 2006) and differential expression of one gene GUCY1A3 (Sabatini et al., 2007) in amygdala of EARE exposed monkeys. However, human studies in maltreated children showed contrary results, with some studies found no volume changes (De Bellis et al., 2001; De Brito et al., 2013; Hanson et al., 2010; Woon & Hedges, 2008), while others revealed decreased volume (Edmiston et al., 2011; Hanson et al., 2015; Luby et al., 2013) or greater volume and elevated response (Lupien et al., 2011; Mehta et al., 2009; Tottenham et al., 2010). Furthermore, those studies found greater volume and elevated response of amygdala (Mehta et al., 2009; Tottenham et al., 2010) were performed several years after the institutionalized children adopted by high socio-economic status families. These data suggested that EARE modified amygdala changes was resistant to recovery, and it was consistent with primate research that suggested abnormal behaviors was resistant to environmental enrichment treatments (Lutz et al., 2004; Lutz & Novak, 2005; Novak et al., 1998; Rommeck et al., 2009a). Similarly, in adults exposed to EARE some studies found no significant changes of amygdala volume (Bremner et al., 1997; Cohen et al., 2006), while others found larger volume (Evans et al., 2016; Lyons-Ruth et al., 2016), interrupted regulation of negative emotion (Kim et al., 2013), increased response to potential rewards (Casement et al., 2014), elevated amygdala responses to threat but not happy faces (Javanbakht et al., 2015). In addition to amygdala structure and activity changes, its connectivity with other brain regions was also affected (Barch et al., 2016; Jedd et al., 2015). Despite those controversial results, the influence of EARE on emotion such as the elevated response to emotion stimuli both in human and primates (Casement et al., 2014; Javanbakht et al., 2015; Nelson et al., 2009) should be mainly achieved through its influence on amygdala.

Hippocampus

Hippocampus, a major component of the brains located inside the medial temporal lobe and beneath the cortical surface, is involved in episodic, declarative, contextual, and spatial learning and memory, as well as being a component in the control of autonomic and vegetative functions (Buckley, 2005; Eichenbaum, 2001; Eichenbaum et al., 1992, 1996; Manns &

Eichenbaum, 2006; Opitz, 2014; Shohamy & Turk-Browne, 2013). In human studies, EARE induced significant reduction of hippocampal volume was an consistent finding in adults (Bremner et al., 1997; Cohen et al., 2006; Hart & Rubia, 2012; McCrory et al., 2011; Stein et al., 1997; Woon & Hedges, 2008). However, children and adolescents studies showed inconsistent results, with few found decreased volume (Edmiston et al., 2011; Hanson et al., 2015; Luby et al., 2013), while most found no significant change (Carrion et al., 2001; De Bellis et al., 2001, 1999, 2002; Mehta et al., 2009; Tottenham et al., 2010; Woon & Hedges, 2008). Primate studies also found no significant hippocampal volume change in PR (Sánchez et al., 1998; Spinelli et al., 2009) and repeated mother deprived (Law et al., 2009b) juvenile monkeys, suggesting changes of hippocampus seemed to happen later in life compared to early life amygdala changes. Two possible explanations could account for the discrepancy of children and adult findings. Firstly, that might due to the fact that the hippocampus develops mainly in the first years of life, therefore less affected by exposure to adversity in childhood and adolescence (Houston et al., 2014; Lenroot & Giedd, 2006; Richards & Xie, 2015). Another possibility is that EARE might not have an immediate effect on the hippocampus but induced changes over time, and long-term effects of EARE exposure may be delayed and became manifest only in later phases of development when the vulnerable brain reaches maturation (Andersen & Teicher, 2004; Brunson et al., 2005; Gluckman & Hanson, 2004; Gluckman et al., 2007; Sapolsky et al., 1985). Moreover, human studies found interesting results concerned with influence of EARE exposure on structure and activity of hippocampus and amygdala, with decreased hippocampal volume and activity in humans exposed to adulthood stress (Bremner et al., 2007; Lupien et al., 2007; Rauch et al., 2000) and adults experiencing EARE (Bremner et al., 1997; Cohen et al., 2006; Stein et al., 1997; Woon & Hedges, 2008), while increased amygdala volume and activity in humans exposed to adulthood stress (Bremner et al., 2007; Lupien et al., 2007; Rauch et al., 2000) and adults experiencing EARE (Mehta et al., 2009; Tottenham et al., 2010). Although the biological mechanism and meaning of this phenomenon remains unclear, that might contribute to or even be the direct reason for the impaired learning and memory ability (decreased hippocampal volume and activity related) and elevated response to emotional stimuli (increased amygdala volume and activity related) described above.

Prefrontal cortex

The prefrontal cortex (PFC) is the anterior part of the frontal lobes of the brain and implicated in planning complex cognitive behaviors, personality expression, decision making and moderating correct social behavior. Children and adolescents studies showed inconsistent results of EARE induced PFC structural changes, with findings of either no significant differences (De Bellis et al., 1999), or significantly smaller volume (De Bellis et al., 2002; Edmiston et al., 2011; Hanson et al., 2010; Morey et al., 2016; Thomaes et al., 2010) or significantly larger volume (Carrion et al., 2009; Richert et al., 2006). In contrast, decreased PFC volume in adults exposed to childhood maltreatment was a consistent finding (Tomoda et al., 2009; van Harmelen et al., 2010). That might due to the fact that PFC continues to develop during adolescence (Houston et al., 2014; Lenroot & Giedd, 2006; Richards & Xie, 2015), therefore might be particularly vulnerable to the effects of stress during adolescence. In addition to the structural changes, EARE could also induce PFC functional changes, with some human adults exposed to EARE showing reduced prefrontal cortex activity during monetary reward anticipation and emotion regulation (Casement et al., 2015; Kim et al., 2013; Romens et al., 2015; Schweizer et al., 2016), while others showing increased response to potential rewards and threatening faces and in passive viewing conditions (Casement et al., 2014; Javanbakht et al., 2015; Jedd et al., 2015; Wang et al., 2016; White et al., 2015). One primate report indicated PR juvenile monkeys showed greater enlarged medial prefrontal cortex (mPFC) size (Spinelli et al., 2009). Moreover, both rodent and primate studies revealed the direct underlying epigenetic mechanisms of EARE on PFC through influencing differential gene expression, histone acetylation and DNA methylation (Blaze et al., 2015a; Provençal et al., 2012; Wall et al., 2012). Studies regarding EARE effects on PFC in primates are rare, and further investigations are necessary.

Other brain regions

The anterior cingulate cortex (ACC) is the frontal part of the cingulate cortex, and appears to play a role in a wide variety of rational cognitive functions, such as reward anticipation, decision-making, empathy and emotion (Devinsky et al., 1995; Drevets et al., 2008). It can be divided anatomically into dorsal and ventral components, with dorsal part connected with PFC making its involvement in cognition possible, and the ventral part connected with amygdala making its involvement in emotion possible (Bush et al., 2000; Morecraft et al., 2007). Human studies showed reduced volume of adult ACC in people with mood disorders (Botteron et al., 2002; Drevets et al., 1997; Yamasue et al., 2003), adults exposed to early life stress (ELS) (Cohen et al., 2006) and abuse-related Posttraumatic stress disorder (PTSD) (Kitayama et al., 2006; Thomaes et al., 2010) and major depressive disorder (Treadway et al., 2009). On the contrary, a primate study found enlarged ACC in PR juvenile monkeys (Spinelli et al., 2009). Moreover, an epigenetic study showed parental separations in infant marmoset affected expression of genes in the ACC of adolescent monkeys (Law et al., 2009a). Additionally, both human and primate studies revealed EARE affected cerebellum, with human studies showing decreased cerebellum (Bauer et al., 2009; Edmiston et al., 2011), while a primate study revealing larger cerebellar vermis area in PR juvenile monkeys (Spinelli et al., 2009). EARE effect on primate cerebellum might due to the fact that macaque cerebellum has high density of glucocorticoid receptors (GRs) (Sánchez et al., 2000), which put it particularly vulnerable to stress hormones related over stimulation. Striatum was another brain region affected by EARE, with increased response to potential rewards (Casement et al., 2014) and elevated dopamine responses to amphetamine (Oswald et al.,

2014), and a potential neurobiological mechanism linking early-life adversity and altered ventral striatal development was indicated (Goff & Tottenham, 2015). In addition to those specific regional changes, PR chimpanzees showed less global white-to-grey matter volume and cortical folding (Bogart et al., 2014). Structural connectivity between different brain regions was also affected by EARE, as studies showed affected corpus callosum, a wide and flat bundle of axons beneath the cortex connecting left and right cerebral hemispheres and facilitating inter-hemispheric communication, in a inconsistent way that most human studies showing EARE reduced corpus callosum (De Bellis et al., 1999; Rinne-Albers et al., 2016; Teicher et al., 2004, 1997), while few showing no significant changes (Mehta et al., 2009). Primate studies also found either decreased corpus callosum size (Sánchez et al., 1998) or no significant changes (Spinelli et al., 2009).

Neurons and glia cells

Neurons are the basic unit of brain. Neuronal network is responsible for the daily cognitive and emotional behaviors. Glia cell is a group of non-neuronal cells that support and protect the neurons in the brain. Rodent studies showed that maternal separation could induce morphological alteration of the apical dendrites of CA3 pyramidal neurons (Kwak et al., 2008); could increase corticotropin releasing factor (CRF)-containing neurons in amygdala (Becker et al., 2007); and could decrease *in vivo* firing activity of amygdala neurons (Adams & Rosenkranz, 2016) and sex related neurogenesis (Oomen et al., 2009). Chronic stress could induce atrophy of dendrites in hippocampus of rats (Brunson et al., 2005; McEwen, 1999) and tree shrews (Magariños et al., 1996), and could induce hippocampal neuroplasticity changes (Fenoglio et al., 2006). Bartesaghi and colleagues used guinea-pig as animal model to investigate the effects of early isolation on neurons, and they found that early isolation could induce morphologic changes of neurons in entorhinal cortex and hippocampus (Bartesaghi et al., 2003a, b; Bartesaghi & Serrai, 2001, 2004). Although primate studies found neuronal morphological changes in EARE exposed monkeys (Bryan & Riesen, 1989; Floeter & Greenough, 1979; Stell & Riesen, 1987; Struble & Riesen, 1978), these early findings were limited to cerebellum, somatosensory and motor cortex, with limited information on other important brain regions, such as hippocampus, amygdala and PFC. Recent studies showed that different environments could induce neuron plasticity changes in the key brain regions involved in learning and memory. Complex environment could enhance complexity of the dendritic tree and density of dendritic spine in hippocampus and PFC in monkeys (Kozorovitskiy et al., 2005). Early parental deprivation in the marmoset monkey could produce long-term changes in hippocampal expression of genes involved in synaptic plasticity and implicated in mood disorder (Law et al., 2009b). So these neuron morphological and plasticity changes might explain and account for how EARE take effects on cell level, and then further more leading to behavioral changes.

As EARE effects on glia cells, rodent studies revealed that EARE could induce long-term changes of astrocyte density and numbers in many brain regions, including PFC, mPFC, hippocampus, cingulate cortex and amygdala (Leventopoulos et al., 2007), and could alter behavioral, autonomic and endocrine responses to environmental challenge (Musholt et al., 2009; Rüedi-Bettschen et al., 2006). Although there was no direct evidence pointing out that glia cell changes were responsible for those altered responses in rats, those studies at least suggested the involvement of glia cell in EARE induced effects. Moreover, human studies showed that glial cell depletion in many brain regions was related to mood disorders, as the number of glia cell was reduced in PFC of both major depressive disorder (MDD) and bipolar disorder (BD) patients (Öngür et al., 1998), in the amygdala of major depressive disorder patients (Bowley et al., 2002) and in anterior cingulate cortex of major depressive disorder and schizophrenia patients (Cotter et al., 2001). Considering the important trophic influence of glia on neurons, glia cell deficits induced by EARE could possibly be responsible for EARE effects on neurons and furthermore to abnormal behavioral function. If that is true, how does it happen? Rodent Studies showed that stress related hormone glucocorticoid receptors (GRs) were also expressed in glia cells (Bohn et al., 1991; Jung-Testas & Baulieu, 1998; Vielkind et al., 1990). Glucocorticoid is the product of the HPA axis, so EARE might take effects through its influence on stress related hormones, like glucocorticoid, and then exert influence on glia cells leading to various effects (Jauregui-Huerta et al., 2010). Indeed, *in vitro* and *in vivo* studies showed that glucocorticoids could influence gene expression in glia cells (Bohn et al., 1994; Kumar et al., 1985) and could regulate the concentration of glial fibrillary acidic proteins (O'Callaghan et al., 1989). By playing central roles in learning and memory, hippocampal astrocyte number was dose-dependently increased by corticosterone treatment (Bridges et al., 2008), and glial responses in hippocampus was also regulated by glucocorticoid through influencing gene expression (Nichols et al., 2005). However, few studies were performed to investigate this issue and EARE affected glia cell changes were link directly to behavioral outcomes without solid evidence. In primate studies, there are lack of evidence to support that EARE affects glia cell structural and functional changes, and furthermore, induces behavioral outcomes.

Lateralisation

Some studies suggested that the influence of EARE on different brain hemisphere might be different, and different type of EARE might take effects differentially on the same brain structure. A human study found that the institutionalized children had greater right amygdala volume, while the left amygdala volume was smaller in the children experienced longer periods of deprivation (Mehta et al., 2009). Another human study showed that patients with child abuse-related complex PTSD showed reduced gray matter concentration in right hippocampus and right dorsal ACC, but not in the left areas (Thomaes et al., 2010). In primate studies, maternal separation was associated with activation in the right dorsolateral PFC and decreased activity in the left dorsolateral PFC of juvenile rhesus monkeys (Rilling et al., 2001). Not only brain structure and function

showed lateralisation affection by early experiences, behavioral research also found lateralisation in primates, as an interesting research showed that PR monkeys demonstrated greater left-hand bias compared to MR reared monkeys (Bennett et al., 2008). The number of lateralisation related studies is limited and the underlying mechanism remains unknown, which certainly adds complexity to the understanding of the influence EARE on brain structure and functional changes and the related abnormal behavioral outcomes.

Other EARE effects

Young animals are particularly vulnerable to EARE effects

Adverse experience has its influence over all life stages, including early, middle and later life, in which infants are especially vulnerable to EARE and the consequences could persistent into later life. That might due to the fact that the most sensitive period of the whole life is the early stage, during which the body is undergoing profound physiological development, such as HPA axis, and brain is also undergoing profound neural development, such as neurogenesis. The amygdala developes rapidly during the early postnatal period in animals, e.g., in rats, cats and primates (Kikusui & Mori, 2009; Lupien et al., 2009; Payne et al., 2010; Wakefield & Levine, 1985). Stress related hormones and receptors were also maximally expressed in the brain early in development (Avishai-Eliner et al., 1996; Baram & Hatalski, 1998; Meaney & Szyf, 2005; Pryce et al., 2005a; Vazquez et al., 2006). These early physiological development heighten the vulnerability of the brain to environmental exposures. On the other hand, the proper development needs proper environmental stimuli, and the natural and best stimuli during early life is the attachment between caregivers, especially mothers, and infants, as mothers could supply tactile contact, physical warmth, nourishment, and psychological comforts. As stated in attachment theory and affectional system, infants need to develop a stable relationship with the mother for social and emotional development to occur normally, while various EARE intervene the forming of the bonds of this relation, therefore both short-term and long-term devastating influence are inevitable.

Sexual differences in EARE effects

Humans studies showed that affectability of various mental disorders were sex-related during development, with boys showing higher tendencies to develop aggression and novelty seeking behaviors (Farrington & Loeber, 2000) while girls more susceptible to anxiety and depression (Kessler, 2003). Additionally, EARE influence might also be sex related, e.g., corpus callosum volume reduction was only found in EARE exposed males (De Bellis et al., 1999). Similarly, animal studies also revealed the vulnerability of males to EARE in rodents (Galea et al., 1997; Kikusui & Mori, 2009) and primates (Clarke, 1993; Cross & Harlow, 1965; Mitchell, 1968; Rommeck et al., 2009a; Suomi et al., 1971). On the contrary, other studies showed preference of EARE on females in humans (Heim & Nemeroff, 2001; Klimes-Dougan et al., 2001), rodents (Hoyer et al., 2013; Ziabreva et al., 2003b) and primates (Sánchez et al., 2005). Previous studies showed that stress could induce decreasing in number and length of apical dendritic branch of medial prefrontal cortex in male rats, whereas increasing in apical dendritic length in female rats (Garrett & Wellman, 2009). Isolated males showed less dendritic branches, shorter dendritic length and smaller dendritic spine density than control males, while isolated females had more dendritic branches than control females in guinea pig (Bartesaghi et al., 2003a). Neurogenesis was significantly increased in male but decreased in female offspring after maternal deprivation in rats (Oomen et al., 2009). The mechanism of those sexual differences remains unclear, but one possible explanation is the gender related physiological differences, such as neuroendocrine system and brain structure and function, which may induce different behavioral and physiological responses in male and female subjects. .

Time effects of EARE

Early life is a time of heightened susceptibility to EARE and expression of adverse experiences induced effects would be different across life time, therefore the time of administration of adverse experiences and subjects age of measurement might partially explain the discrepant findings across studies (Tottenham & Sheridan, 2009).

The time of adverse experiences administration is important, as different brain regions might have unique windows of vulnerability to stress, e.g., human studies indicate that the time window of hippocampus, corpus callosum and frontal cortex is at ages of 3-5, 9-10 and 14-16 years, respectively (Andersen et al., 2008). Rodent studies revealed the critical importance of specific time windows early in life for the outcome of maternal separation (Bock et al., 2005; Gos et al., 2008; Pryce et al., 2005b). Early primate studies by Harlow et al. showed the importance of administration time of adverse experiences (isolation), in a way that isolation beginning at birth generated most severe effects and persisting abnormalities (Harlow et al., 1965; Mitchell, 1968), while the isolation starting until later in life would produce less severe effects and persistent abnormalities (Harlow et al., 1965; Mitchell, 1968). Moreover, different lasting period of EARE also produced different effects even was all initiated at birth, i.e., 3 months isolation only induced reversible debilitating behavioral deficits, while at least six months isolation generated most severe effects and persisting abnormalities; 3 months isolation induced least, 6 months isolation induced moderate and 12 months isolation induced most severe defects (Griffin & Harlow, 1966; Harlow et al., 1965; Mitchell, 1968). These studies suggested that both the time point of administration of EARE and the lasting period have different influences on behavioral and biological outcomes.

Human studies showed different, or even contrary effects of EARE in children and adults, suggesting EARE might induce differential outcomes across lifespan. For example, childhood abuse induced significant reduction of hippocampal volume in adults (Bremner et al., 1997; Cohen et al., 2006; Stein et al., 1997; Woon & Hedges, 2008) but not in children (Carrion et al., 2001; De Bellis et al., 2001, 1999; Woon & Hedges, 2008); EARE induced hypercortisolism in children (Essex et al., 2002;

Fernald & Gunnar, 2009; Flinn & England, 1997; Kaufman et al., 1997) but hypocortisolism in adults (Carpenter et al., 2009; Elzinga et al., 2008); adults with abuse related PTSD showed ACC volume reductions (Kitayama et al., 2006; Thomaes et al., 2010), whereas pediatric PTSD showed increased ACC (Richert et al., 2006). Primate studies also found similar results, e.g., monkeys exposed to EARE showed less aggression during infancy (Chamove et al., 1973; Harlow et al., 1965) but more aggression during latter life (Chamove et al., 1973; Mitchell, 1968), whereas showed more affiliative behavior during infancy (Chamove et al., 1973; Rosenblum & Paully, 1984; Ruppenthal et al., 1991) but less during adulthood (Kraemer & Mckinney, 1979; Rosenblum & Paully, 1984; Winslow et al., 2003). Monkeys exposed to EARE showed more activity during infants (Champoux et al., 1991) but less activity during adulthood (Harlow & Suomi, 1971a; Mason & Sponholz, 1963; Mitchell, 1968); The number and style of stereotypies exhibited in monkeys also varied by age, e.g., the number of whole-body stereotypies were negatively correlated with age, whereas self-directed stereotypies were positively correlated; moreover younger monkeys exhibited more pacing, body-flipping, and swinging, while older ones exhibited more hair-pulling and saluting (Lutz et al., 2003). These studies showed different, or even opposite effects of EARE on behavioral and biological outcomes between infants and adults, indicating EARE induce different outcomes across lifespan.

MECHANISMS UNDERLYING EARE INDUCED EFFECTS

Neuroendocrinological mechanisms

Some recent study linked behavioral outcomes with EARE affected neuroendocrine systems, and suggested that EARE might modulate subsequent social behaviors through regulating both the production and body's sensitivity to neurotransmitters and hormones (Cushing & Kramer, 2005). Moreover, studies indicate that the involved neurotransmitters and hormones were mainly monoamine neurotransmitter serotonergic systems, including serotonergic system and catecholamine system (both noradrenergic system and dopaminergic system), and glucocorticoid hormones (cortisol in non-human primates and humans), oxytocin and growth hormone (GH)(Table 4).

Table 4 EARE induced effects on neuroendocrine systems

	Outcome		Primate studies	Human studies
Serotonin system	PR and SPR monkeys showed decreased CSF levels of 5-HIAA		Fahlke et al., 2000; Higley et al., 1996a; Maestripieri et al., 2006; Shannon et al., 2005	
	PR monkeys showed decreased SERT binding potential		Ichise et al., 2006	
	VFD monkeys were hyporesponsive to the serotonergic probe mCPP		Rosenblum et al., 1994	
Catecholamine system	PR monkeys showed lower CSF concentrations of HVA and attenuated NE secretion		Clarke et al., 1999; Clarke et al., 1996	
	VFD monkeys were hyper responsive to the noredrenergic probe yohimbine		Rosenblum et al., 1994	
	PR monkeys showed significantly lower DOPAC concentrations		Clarke et al., 1999; Clarke et al., 1996	
HPA axis dysregulation	Hypercortisolism	Hormone level	Barrett et al., 2009; Coplan et al., 2005; Coplan et al., 1996; Coplan et al., 2001; Suomi, 1991	Essex et al., 2002; Flinn & England, 1997; Gunnar et al., 2001; Kaufman et al., 1997
		Increased HPA response	Fahlke et al., 2000; Higley et al., 1992; Kraemer et al., 1983, 1984; Sánchez et al., 2005; Suomi, 1991	Heim et al., 2000b; Kikusui & Mori, 2009; Pesonen et al., 2010
	Hypocortisolism	Hormone level	Clarke et al., 1998; Capitanio et al., 2005; Shannon et al., 1998	Brand et al., 2010; De Bellis et al., 1994; Heim et al., 2000a
		Decreased HPA response	Barr et al., 2004; Capitanio et al., 2005; Clarke, 1993; Dettling et al., 1998; Dettling et al., 2002a, b; Lyons et al., 2000; Parker et al., 2004	Carpenter et al., 2009; Elzinga et al., 2008; Hart et al., 1995

Monoamine and hormone systems

The serotonergic system was shown to moderate the effects of EARE on the risk of depression in humans (Eley et al., 2004; Kaufman et al., 2004), and primate studies also indicate the role of serotonin system in regulating the effects of EARE. Maternal rejected, PR and SPR reared infant monkeys exhibited lower CSF 5-HIAA concentrations (Fahlke et al., 2000; Higley et al., 1996a; Maestripieri et al., 2006; Shannon et al., 2005); PR monkeys showed decreased SERT binding potential across a

range of brain areas (Ichise et al., 2006); VFD reared monkeys were hyporesponsive to the serotonergic probe meta-Chlorophenylpiperazine (mCPP) (Rosenblum et al., 1994). Moreover, epigenetic studies also indicate the role of serotonin system plays in EARE induced HPA axis dysfunction (Barr et al., 2004; Rosenblum et al., 1994; Shannon et al., 2005; Spinelli et al., 2007) and subsequent abnormal behavioral outcomes (Barr et al., 2003, 2004; Law et al., 2009b; Maestripieri et al., 2006; Vicentic et al., 2006; Ziabreva et al., 2003a). Many studies showed catecholamine system is another candidate through which EARE takes its effect. PR monkeys showed attenuated Norepinephrine (NE) secretion (Clarke et al., 1999, 1996) and reduced CSF concentrations of catecholamine metabolite (Clarke et al., 1999, 1996), while VFD reared monkeys were hyper responsive to the noredrenergic probe yohimbine (Rosenblum et al., 1994). It was further suggested that EARE might influence the differentiation of noradrenergic neurons and thus alter HPA responses stress during adulthood (Liu et al., 2000). Dopamine system (another catecholamine system) might also be involved in EARE effects, as significantly lower concentrations of dopamine metabolite were revealed in PR infant monkeys (Clarke et al., 1999, 1996) and history of childhood adversity was positively associated with striatal dopamine responses to amphetamine (Oswald et al., 2014).

Additionally, primate studies indicate potential hormonal pathways through which EARE takes effects, including oxytocin, growth hormone (GH), and most importantly, cortisol. Monkeys exposed to EARE showed abnormal aggressive and affiliative behaviors, and oxytocin was suggested to be a neuropeptide for affiliation and involved in the regulation of social bonding behaviors (Insel, 1992; Lim & Young, 2006). Therefore, oxytocin is a possible pathway for EARE to take effects, which indeed was probed by Winslow et al. (Winslow et al., 2003), showing that the decrease in affiliative behavior in PR rhesus monkeys was significantly and positively correlated with cerebrospinal oxytocin. Another hormone, GH, was also related to early adversity, as PR and social separation experiences in infant monkeys showed abnormal GH levels (Champoux et al., 1989a; Laudenslager et al., 1995). Most importantly, the main target of EARE under investigation is HPA axis. While some studies showed blunt HPA response, and thus decreased cortisol and ACTH levels (Barr et al., 2004; Capitanio et al., 2005; Clarke, 1993; Dettling et al., 1998, 2002a, b; Lyons et al., 2000; Parker et al., 2004), others showed the opposite (Barrett et al., 2009; Coplan et al., 2005, 1996, 2001; Suomi, 1991). Although consistent results were not achieved, the importance of HPA dysregulation in EARE induced effects was suggested.

Hypothalamic-pituitary-adrenal (HPA) axis dysregulation

EARE is associated with elevated levels of stress and fear. The adverse impact of stress on brain development was suggested to be largely through hypothalamic-pituitary-adrenal (HPA) axis both in humans (Loman & Gunnar, 2010) and primates (Sanchez, 2006). The effects of EARE on HPA circadian rhythmicity and the function of HPA axis were reviewed in this section.

Circadian rhythmicity

TheHPA axis is a complex set of direct influences and feedback interactions among three endocrine glands, i.e., hypothalamus, pituitary gland, and adrenal glands. Under basal conditions, HPA axis exhibits a circadian rhythmicity with a peak around the time of waking and a trough during the quiescent time of the activity cycle (Dickmeis et al., 2013; Leliavski et al., 2015; Tsang et al., 2016, 2014). So cortisol levels typically follow the circadian rhythm with levels highest occurring about 20 minutes after awakening in the morning (cortisol awakening response, CAR) and declining throughout the day. Alterations in the normal pattern of HPA rhythmicity, including CAR response and diurnal decrease of cortisol, were found in human studies. Most studies found higher morning cortisol level than controls in maltreated children (Cicchetti & Rogosch, 2001; Cutuli et al., 2010) and EARE exposed adults (Gonzalez et al., 2009; Gustafsson et al., 2010), while some found lower morning cortisol level (Carlson & Earls, 1997). Moreover, different kind of EARE might have differential influence on morning cortisol values, as studies indicate more emotionally and sexually abused children showed higher morning cortisol values, whereas more severe physically neglected and abused children showed lower levels (Bruce et al., 2009; Cicchetti & Rogosch, 2001). Additionally, EARE exposed children also showed higher incidences of atypical diurnal rhythmicity patterns, such as a peaking in the afternoon or evening (Cicchetti et al., 2010; Dozier et al., 2006). Similarly, abnormal HPA circadian rhythmicity were also found in limited amount of primate studies on rhesus monkeys, with morning peak occurring late in PR infants (Thomas et al., 1995) and flattened diurnal rhythm in repetitive maternal separation exposed infants (Sánchez et al., 2005). However, a recent study found no shift in diurnal patterns of cortisol in PR reared juvenile rhesus monkeys (Barrett et al., 2009). Although how EARE induces those abnormal HPA axis circadian rhythmicity, and its different or even contrary effects remains unknown, these HPA axis circadian rhythmicity abnormalities certainly contribute to various abnormal behavioral outcomes.

HPA axis dysregulation

In humans, the HPA axis develops over the initial several years of life and is highly sensitive to EARE (De Weerth et al., 2003; Watamura et al., 2004). The key elements of the HPA axis are as following: the hypothalamus synthesizes and secretes corticotropin-releasing hormone (CRH); CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) in pituitary gland; ACTH acts on the adrenal cortices, which then produces glucocorticoid hormones (mainly cortisol in NHPs and humans); glucocorticoids in turn act back on the hypothalamus and pituitary to suppress CRH and ACTH production in a negative feedback cycle. When activated in response to a stressor, the HPA axis participates in a cascade of neuroendocrine responses, and a typical HPA stress response involves a period of increased glucocorticoids in circulation induced by stimulation of elevated levels of CRH and ACTH, followed by a return to baseline levels induced by negative feedback of glucocorticoids

(Herman & Cullinan, 1997). Thus CRH, ACTH and glucocorticoids levels could indicate the reactivity levels of HPA axis, with CRH an important neurotransmitter in HPA axis to initiate the autonomic and behavioral changes in response to stress (Heinrichs et al., 1995; Krohg et al., 2008; Ohmura & Yoshioka, 2009; Smagin & Dunn, 2000). Studies showed EARE induced elevated cerebrospinal fluid (CSF) concentrations of CRH levels in mother deprived rats (Ladd et al., 1996) and VFD reared infant monkeys (Coplan et al., 1996). Not only the CRH levels was increased, a study showed that EARE increased the density of CRH binding sites in many brain regions, including PFC cortex, amygdala and hippocampus (Anisman et al., 1998). As analysis of CRH requires sampling of CSF, it was hard to perform the experiment on healthy humans. On the other hand, analysis of ACTH and glucocorticoids (cortisol) only requires blood or urine sampling, so they are more widely investigated in humans.

Glucocorticoids was revealed to be released from the adrenal cortex during neuroendocrine responses to stress (Herman et al., 2003, 1996), and then regulate HPA axis via negative feedback by binding to two types of receptors, mineralocorticoid receptors (MRs) with high affinity (important in proactive maintenance of HPA basal activity), and GRs with low affinity (primarily responsible for negative feedback). Glucocorticoids could pass through the blood-brain barrier to influence brain function (Zarrow et al., 1970), and MRs' expression was significantly greater in monkey infants than other ages (Pryce et al., 2005a). Therefore, HPA axis was one of the major pathways through which EARE induces stress and shapes brain development, particular in infants. Glucocorticoids could facilitate HPA axis activation by occupying its receptors in amygdala, leading CRH increase within amygdala (Kolber et al., 2008), whereas it could also suppress HPA axis by occupying its hippocampal receptors (van Haarst et al., 1997). Amygdala and hippocampus are important brain regions for socio-emotional functioning and learning and memory throughout development, and they have a high density of receptors for unbound glucocorticoids, therefore are major targets of EARE affected HPA axis (Johnson et al., 2005; Sánchez et al., 2000). Additionally, EARE could affect HPA axis function bidirectionally, with some studies showing attenuated basal and challenge induced levels of cortisol (hypocortisolism), while others showing elevated levels in both conditions (hypercortisolism).

Hypercortisolism

Human studies showed that EARE could induce hypercortisolism of basal cortisol level in children, reflected by elevated levels of cortisol (Essex et al., 2002; Flinn & England, 1997; Gunnar et al., 2001) and ACTH (Kaufman et al., 1997) in EARE exposed children. Primate studies also showed EARE induced hypercortisolism, reflected by increased plasma coritsol and ACTH in PR infants and juvenile monkeys (Barrett et al., 2009; Suomi, 1991). The elevated cortisol levels in hairs of PR infants indicate the long time accumulation of EARE outcomes (Dettmer et al., 2012). Increased basal cortisol levels were found to be induced by prenatal stress (Pryce et al., 2011). Persistently elevated CSF concentrations of CRF in both infants and mothers under VFD conditions were reported (Coplan et al., 2005, 1996). Not only EARE could affect infants and children, it was suggested that childhood abuse was associated with a persistent sensitization of the HPA axis to stress in human adults (Elzinga et al., 2008), e.g., adults exposed to EARE had higher HPA reactivity during the Trier Social Stress Test (TSST) (Heim et al., 2000b; Pesonen et al., 2010). Animal studies also showed hyper-response of HPA axis activity when facing stress in both infants and adults exposed to EARE. Rodent studies revealed higher HPA response to novelty stress in early-weaned mice (Kikusui & Mori, 2009). Primate studies showed hyper-responsiveness in EARE reared monkeys, reflected by increased cortisol response to stress in monkeys exposed to PR rearing (Fahlke et al., 2000; Suomi, 1991), VFD rearing (Coplan et al., 2001), repetitive maternal separation (Sánchez et al., 2005) and parental deprivation (Higley et al., 1992; Sánchez et al., 2005). Amphetamine challenge test also revealed neurochemical and behavioral hyper-responseness in isolated monkekys (Kraemer et al., 1983, 1984). All those studies suggested EARE induced hypercortisolism, reflected by elevated basal and stress or challenge facing levels of cortisol, ACTH or CRF.

Hypocortisolism

EARE induced hypocortisolism was also a common finding (Gunnar & Vazquez, 2001), e.g., maltreated children showed decreased basal levels of cortisol (Brand et al., 2010; Heim et al., 2000a) and ACTH (De Bellis et al., 1994). Primate studies revealed attenuated basal levels of cortisol and ACTH in PR monkeys (Capitanio et al., 2005; Clarke et al., 1998; Shannon et al., 1998). When facing stress or challenge, EARE exposed children and adults both showed blunt cortisol response and thus reduced cortisol level (Carpenter et al., 2009; Elzinga et al., 2008; Hart et al., 1995). Similarly, primate studies showed blunt HPA responses during stress and thus decreased coritsol and ACTH levels in PR reared infants (Barr et al., 2004; Capitanio et al., 2005; Clarke, 1993), in young adults exposed to maternal deprivation and intermittent separation (Capitanio et al., 2005; Lyons et al., 2000) and in the hairs of PR infants (Feng et al., 2011) of rhesus monkeys. EARE induced hypocortisolism was also found in other monkey species, including maternal neglect exposed juvenile Goeldi's monkeys (Dettling et al., 1998), intermittent stress exposed squirrel monkeys (Parker et al., 2004) and parental deprivation exposed marmosets (Dettling et al., 2002a, b). All those studies indicate hypocortisolism reflected by decreased basal and stress or challenge facing levels of cortisol or ACTH.

As described above, it is controversial as to the effects of EARE on HPA axis, with some studies showing hypercortisolism while other showing hypocortisolism. There are several possible reasons. Firstly, different types of EARE vary between different research, and even for a same type of EARE, the procedures, manipulations, tests and measuring indexes could be different in different experiments. Secondly, different genotype among human races or animal species could contribute to the divergence as well, in a way that individuals with certain

genotype may be more sensitive to a particular type of EARE than others. In addition, subjects' personality or temperaments could also partially contribute to the divergence, e.g., children with inhibited temperaments tended to have higher cortisol levels than extroverted children (Gunnar et al., 1995; Kagan et al., 1988), indicating that long-term consequences of EARE may not uniform across subject populations..

A sample of EARE induced neurotransmitter and hormonal changes related behavioral outcomes - social status of primates

Studies suggest that EARE could induce abnormal changes of neurotransmitters and hormones and then influence social status of primates. Serotonergic system was the most widely studied neurotransmitter involved. Primate studies showed that different levels of CSF serotonin (5-HT) or its main metabolite 5-Hydroxyindoleacetic acid (5HIAA) were related to different social status, with higher levels related to more dominant status (Higley et al., 1996b; Raleigh et al., 1983). Additionally, serotonergic drugs were found to be able to influence dominance status, in a way that serotonergic enhancing drugs increase social dominance while serotonergic reducing drugs decrease dominance (Raleigh et al., 1991). 5-HT seems to be positively related to social dominance status, and studies suggested that might due to its influence on affiliative and aggressive behaviors which are important factors in dominance formation and maintenance. Primate studies showed positive correlation between CSF 5-HIAA and affiliative approaching and grooming behavior (Mehlman et al., 1995; Raleigh et al., 1985) and negative correlation between CSF 5-HIAA and aggressive behavior (Higley et al., 1996a), which were consistent with the previous presumption that affliliative behavior was much more effective in acquiring and reinforcing social dominance than aggressive behaviors. Supporting evidence also came from another genetic primate study that suggested certain serotonin transporter (5-HTT/SERT) diplotypes might modulate acquisition of dominance status (Miller-Butterworth et al., 2007). Beside serotonergic system, dopaminergic systems might also affect social dominance status, as dopamine transporter (DAT) gene variants were suggested to be associated with social rank in cynomolgus monkeys (Miller-Butterworth et al., 2008). As to hormones, although a study showed that cortisol concentration was significantly higher in dominant monkeys (Czoty et al., 2009), most studies failed to find the relationship between cortisol level and social rank (Czoty et al., 2009; Goo & Sassenrath, 1980; Morgan et al., 2000; Stavisky et al., 2001). Those studies suggested that EARE could influence social status of primates through its influence on neurotransmitters and hormones. The effects of EARE should not be limited on social status but also might on some other abnormal behaviors.

Genetic and epigenetic influences of EARE effects

Developing is a dynamic process involving constant and reciprocal interactions between organisms and the environments. Emerging evidence suggests that epigenetic modifications may serve as a critical mechanism through which experiences occurring during the lifespan can have sustained effects in developmental outcomes (Daskalakis et al., 2013). Epigenetics refers to the study of inherited changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence, such as modifications of transcription of the genome by chemical markers regulation, and variation in gene expression rather than gene sequence is the key concept. Moreover, epigenetics is used to describe the dynamic interactions between genome and the environment (Jablonka & Lamb, 2002). Research suggested that environmental events can modify the epigenetic status of the genome by activating intracellular pathways to regulate interaction between transcription factors and their DNA binding sites, leading to changes in gene expression and eventually different levels of proteins (Bagot & Meaney, 2010; Zhang & Meaney, 2010). This is the biological basis for the interplay between environmental factors and the genome in the regulation of individual differences in behaviors and cognition. Both animal and human studies suggest that EARE can lead to lasting changes in neurotransmitter systems and brain function, and then induce cognitive and behavioral changes. However, there was remarked inter-individual variations in responses to adversity (Collishaw et al., 2007; Rutter, 2007), and these variations might be due to different genotype, different living environment and interaction between the genome and environment.

Genetic influences

Different genotype could induce different behavioral outcomes. Allelic variation of the monoamine oxidase A (MAOA) gene was implicated in aggressive behaviors (Volavka et al., 2004). Both human (Caspi et al., 2002; Craig, 2005; Kim-Cohen et al., 2006) and primate (Karere et al., 2009) studies showed that genotype conferring low MAOA activity was related to mental health problems. These findings may partially explain the variability in developmental outcomes associated with maltreatment, e.g., why not all victims of maltreatment grow up to with abnormal behaviors like antisocial problems, and they provide epidemiological evidence that genotypes can moderate children's sensitivity to environmental insults. Similar results was revealed in 5-HTT genotype, as short promoter region of the serotonin transporter (5-HTTLPR) allele was related to increased anxious behavior in primates (McCormack et al., 2009) and highest emotional problem scores in human (Kumsta et al., 2010). Those evidences suggest the importance of genotype in behavioral outcomes.

Epigenetic influences of EARE on gene expression

Environmental and life experience could exert influences on gene expression and time course analysis indicate that maternally induced epigenetics might emerge during the postnatal period and could sustain into adulthood (Weaver et al., 2004). Epigenetic regulation of gene expression is particularly important during the early stages of development, and it is one of the main mechanisms mediating the long-term effects of maternal care on development (Champagne, 2008; Champagne & Curley, 2009; Diorio & Meaney, 2007; Meaney, 2001; Zhang et al., 2006). For example, rodent studies showed that postnatal

maternal licking/grooming (LG) behavior could induce increased hippocampal GR expression (Caldji et al., 1998; Francis et al., 1999; Liu et al., 1997; Weaver, 2007), while low levels of LG during neonates led to reduced expression of estrogen receptor in hypothalamus and reduced response to estrogen (Champagne et al., 2001, 2006, 2003). As to the effects of EARE on gene expression, isolation attenuated social interaction induced gene expression in rodents (Ahern et al., 2016; Lukkes et al., 2012, 2013; Shishkina et al., 2015; Wall et al., 2012). A human study also showed EARE related down regulation of genes containing GR response elements (Miller et al., 2009). In primate studies, early maternal separation could lead to gene expression changes in many brain regions, including differential expression of gene GUCY1A3 in amygdala, decreases in hippocampal growth associated protein 43 (GAP-43) mRNA and 5-HT receptor mRNA (Law et al., 2009b) and a selective long-term effect on expression of genes in ACC (Law et al., 2009a). Moreover, epigenetics is not a binary response across the whole brain. Different genes in different brain regions can be affected in different ways, e.g., early maternal deprivation could either induce reduction of gene expression (Liu et al., 1997; Roceri et al., 2002) or up-regulation of gene expressions (Plotsky et al., 2005; Ziabreva et al., 2000).

Epigenetic influences of EARE on neurobiological and behavioral outcomes

Epigenetic influences on gene expression may lead to different expression patterns of proteins, thus different levels of hormones and neurotransmitters, ultimately lead to different behavioral outcomes. Studies suggested that EARE might exert its effects on behavioral outcomes independent of genotype. Primate studies revealed the importance of environment and life experience independent of genotype. MR monkeys showed significantly up-regulated level of 5-HTT during maternal separation, while NR monkeys did not (Kinnally et al., 2008). With the same low-activity Monoamine oxidase A (MAOA) genotype, MR reared monkeys were more aggressive than the PR monkeys (Newman et al., 2005). Higher 5-HTT cytosine-phosphate-guanosine (CpG) methylation, but not rh5-HTTLPR genotype, exacerbated the effects of early life stress on behavioral stress reactivity in infant monkeys (Kinnally et al., 2010). Additionally, from behavioral perspective alone, infant monkeys exposed to mother abuse showed the same tendency to their offspring, regardless of whether they were reared by their biological mothers or by foster mothers (Maestripieri, 2005). Rodent studies also supported the important role of environment and life experience on behavioral outcomes. Rodent studies suggested that maternal care behaviors especially postnatal maternal LG could be transmitted from the mother to her female offspring, so female offspring who received low levels of LG also provided low levels of this form of maternal care to their own offspring (Fleming et al., 2002). Cross-fostering studies in rodents indicate that this intergenerational transmission of behaviors was the result of early experience rather than genetic inheritance (Champagne & Meaney, 2001). For example, the biological offspring of low-LG mothers reared by high-LG dams resembled the normal offspring of high-LG mothers (Francis et al., 1999). All those studies indicate the importance of environment and life experience, especially maternal interactions on the subsequent expression of behaviors, rather than the genetic contributions. Moreover, primate studies showed the importance of the interaction between genetic factors and environmental experience on neurobiological outcomes, as CSF 5-HIAA concentrations were significantly influenced by genotype in the PR but not MR reared monkeys (Bennett et al., 2002), and 5-HTT gene variation affected HPA axis activity in response to stress in a way that cortisol levels increased during separation in MR but decreased in PR monkeys (Barr et al., 2004).

Transgenerational epigenetic programming

Among the epigenetic mechanisms of EARE, such as DNA methylation, histone modifications, and micro-RNA expression, DNA methylation was the most intensively studied epigenetic phenomenon (Babenko et al., 2015; Blaze et al., 2015b; Jawahar et al., 2015; Provençal et al., 2015; Vaiserman, 2015a, b; Zheng et al., 2014). Actually, not only EARE exposure of the offspring themselves could lead to long time biological and behavioral outcomes, EARE exposure of parents could also influence their offspring, which was defined as transgenerational epigenetic programming phenomenon and has drawn much attention recently. Related studies suggested the underlying epigenetic mechanisms of maternal transgenerational influence to be DNA methylation, histone modifications, and micro-RNA expression (Babenko et al., 2015; Bale, 2014; Blaze & Roth, 2015; Gröger et al., 2016; Miska & Ferguson-Smith, 2016; Nagy & Turecki, 2015).

In addition to maternal influences, fathers can exert influences on offspring development either through direct care in living social environment, or indirectly through interacting with maternal influences. Some recent studies showed the important influence of paternal early experiences on infant development through non-social mechanisms, even in the absence of direct contact with offspring. This emerging field focuses on how environmental influences can epigenetically alter paternal sperm DNA methylation, histone modification and micro-RNA expression, and ultimately change the phenotype and behavior of offspring (Braun & Champagne, 2014; Curley et al., 2011; Day et al., 2016; Kinnally & Capitanio, 2015; Rodgers et al., 2013; Yuan et al., 2016).

DISCUSSION

Rehabilitation

Human studies indicate the possibility of rehabilitation of EARE induced deficits, e.g., Fisher et al. (2000, 2006, 2007) suggested that the improvements of caring following EARE had the potential to prevent or reverse EARE induced HPA axis dysfunction, such as normalizing perturbed diurnal cortisol patterns and reducing basal salivary cortisol level (Fernald & Gunnar, 2009). In primate studies, total social isolation was once considered to induce permanent defect, which was described as learning deficit in some studies, because isolated monkeys were lack of physical interactions and had no

opportunity for social learning with conspecifics, or to gradually develop sophisticated social behaviors (Mitchell, 1968; Sackett, 1969; Suomi et al., 1974). These abnormities might be rehabilitated by socializing the isolate monkeys with conspecifics. Some studies reported that after the isolated monkeys was paired with “therapist" monkeys, less self-directed disturbance activities, or stereotypic behaviors, but more social contact and exploratory behaviors were observed (Harlow & Suomi, 1971b; Suomi, 1973). Less severe self-injurious behaviors were found when isolated monkeys were reared with surrogates (Brunelli et al., 2014; Harlow & Suomi, 1971b) or social housing (Lutz & Novak, 2005). Additionally, environmental enrichment treatments were used to eliminate abnormal behaviors and to normalize the behavioral repertoire of EARE exposed monkeys (Lutz & Novak, 2005; Novak et al., 1998; Rommeck et al., 2009a). It was suggested that environmental enrichment devices could only ameliorate less severe forms of abnormal behavior but not more severe forms of self-injurious or non-injurious self-abuse behaviors (Rommeck et al., 2009a). Those studies indicate rehabilitation is possible, at least partially, but it requires combination of multiple rehabilitation methods, such as socializing, environmental enrichment, and considerable time and effort.

Establishing NHP mental disorder models with EARE methods

EARE exposed NHPs showed signs of various mental disorders, including anxiety, autism and depression etc., making it a potential animal model to study human mental disorders, among which depression model was the mostly investigated one (Gilmer & McKinney, 2003; Pryce et al., 2005b; Worlein, 2014). The influence of EARE on NHPs is through daily life and accumulates over a period of time, making it a more natural model of mental disorders than those induced by drugs or invasive surgeries. The diagnosis of mental disorders in humans usually depends on questionnaire investigation and verbal communication between patients and doctors, which are not doable in NHPs. Therefore NHPs studies usually include daily group living or single subject observation and behavioral analysis, biochemical index analysis (e.g., hormones), brain structural and functional changes analysis by using modern imaging methods, However, due to the complexity of mental disorders, it is very difficult to diagnose mental disorders in NHPs. Different disorders might show very similar behavioral symptoms and biochemical abnormalities, therefore additional indexes are necessary in diagnosing. For example, in NHPs, anxiety and depression share symptoms of stereotypic behaviors and elevated cortisol level in response to stressor, so, more depression specific symptoms, such as lacking of responses to stimulus are needed for diagnosing. It is difficult to differentiate if a monkey was depressed or autistic as in both cases, preference of staying away from social activity and being alone in a corner would be shown. Certain metal disorders could be divided into many subtypes, e.g., depression includes unipolar, bipolar and atypical depression, etc, which certainly adds more complexity in establishing NHP animal models. These might explain the fact that despite being an ideal and irreplaceable animal model, studies on EARE induced NHP mental disorder models are limited.

CONCLUDING REMARKS

In summary, as an irreplaceable animal model, NHP EARE experiments were performed for over 60 years and revealed important insights into understanding the effects of EARE on development and underlying mechanisms of related physiological and psychological diseases. Although much has been learned to date, there is much more to understand about EARE impact on developmental trajectory. Now, with the help of emerging cutting edge technologies, such as new brain imaging method, gene modification, optogenetics, etc, future EARE studies will further clarify these issues and help to cure the diseases.

ACKNOWLEDGEMENTS

The author wishes to thank Prof. Yuan-Ye Ma and the anonymous reviewers and editors of Zoological Research for their valuable opinions and suggestions.

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ZOOLOGICAL RESEARCH

Does mRNA structure contain genetic information for regulating co-translational protein folding?

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ABSTRACT

Currently many facets of genetic information are ill-defined. In particular, how protein folding is genetically regulated has been a long-standing issue for genetics and protein biology. And a generic mechanistic model with supports of genomic data is still lacking. Recent technological advances have enabled much needed genome-wide experiments. While putting the effect of codon optimality on debate, these studies have supplied mounting evidence suggesting a role of mRNA structure in the regulation of protein folding by modulating translational elongation rate. In conjunctions with previous theories, this mechanistic model of protein folding guided by mRNA structure shall expand our understandings of genetic information and offer new insights into various biomedical puzzles.

Keywords: Translational elongation rate; Protein folding; mRNA secondary structure; Codon usage bias

INTRODUCTION

With regards to the full contents of genetic information, the answer to this fundamental question in biology has been frequently updated as newly emerging techniques and growing data constantly challenge our existing understandings (Ramos & Laederach, 2014). On one hand, novel functions of non-coding genome are uncovered (ENCODE Project Consortium, 2012). On the other hand, our understandings of genetic information in coding regions have also extended beyond canonical schemes of how protein folding is regulated within cells.

Commonly known as “the second half of genetic code”(Kolata, 1986), a vast pool of information is required for ensuring correct folding of polypeptide into its native structure. However, little is known about how information stored in nucleotide sequence is transmitted from genome into polypeptide chain. According to the Central Dogma, messenger RNA is frequently targeted for searching regulatory signals for protein folding. Indeed, as evidenced by unequal usage of synonymous codons and its correlation with efficiency and/or accuracy of translational elongation (Gingold & Pilpel, 2011), mRNA molecule obviously contains more information than primary protein sequence. This logic and decades of genomic sequencing have elucidated the association between codon usage bias and protein structures (Spencer & Barral, 2012; Tsai et al., 2008).

Nevertheless, secondary structure of mRNA is often overlooked, probably due to a lack of scalable experiments for detecting RNA structure (Eddy, 2014) and the complexity of *in silico* prediction for the structure of ribosome-bound mRNA. Recently, this viewpoint has altered due to the latest technical innovations, especially ribosome profiling (Ingolia et al., 2009) and several high-throughput assays for mRNA secondary structure (Graveley, 2016). As a result, a novel regulatory role of mRNA structure on protein folding emerges.

In this review, current models of co-translational protein folding were reviewed for elucidating the generic molecular mechanism for its linkage to mRNA structure. Several preliminary studies that correlate computationally predicted mRNA structures with protein conformation shall be discussed. A major focus was placed upon regulatory signals in major mRNA coding sequences rather than a specific mRNA fragment such as translational ramp at 5’ end (Tuller et al., 2010). Furthermore, biologically relevant interpretations of this regulation were offered.

GENETIC INFORMATION GUIDING PROTEIN FOLDING

The complexity of protein folding have been conventionally summarized as the Levinthal’s paradox. It states that the number of possible conformations of a small protein (around 100 residues) was so large that it would require more time than

Received: 01 December 2016; Accepted: 04 Janurary 2017

Foundation items: This study was supported by the start-up grant from “Top 100 Talents Program” of Sun Yat-sen University to JRY (50000-31131114) and General Program of National Natural Science Foundation of China to JRY (31671320)

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DOI:10.13918/j.issn.2095-8137.2017.004

the lifespan of universe (10^{16} seconds) to explore all possibilities and choose the native conformation (Zwanzig et al., 1992). How this astronomic eternity is reduced to biologically feasible range has been a long-standing puzzle of protein folding. As a theoretic milestone of protein folding, the Anfinsen's dogma, also known as thermodynamic hypothesis, suggests that protein conformation is solely determined by its amino acid sequence. In other words, assuming the validity of the Anfinsen's dogma, accurate protein folding requires no additional genetic input other than primary protein sequence. Nevertheless, multiple exceptions to the Anfinsen's dogma were detected later, including but not limited to prion (Fraser, 2014) and kinetically stable proteins (Xia et al., 2007). It has confirmed the existence of regulatory signals that guide protein folding, including *trans* factors such as chaperones and *cis* factors such as codon optimality (see below).

There are two major modes of protein folding. On the one hand, protein folding occurs after the entire coding sequence has been fully translated. Upon dissociation of mRNA with synthesized polypeptide, a generic molecular mechanism of conveying regulatory signals in mRNA into a remote polypeptide chain is unlikely, if not impossible. On the other hand, nascent polypeptide folds co-translationally while it is being synthesized. Many reports suggested that folding of many proteins was at least partially co-translational (Hardesty et al., 1999; Pechmann et al., 2013; Rodnina & Wintermeyer, 2016). More importantly, mRNA, ribosome and nascent polypeptide form a complex, allowing the transmission and/or realization of regulatory signals in mRNA for protein folding (Pechmann et al., 2013). In fact, current models linking mRNA structure to protein folding are all based upon co-translational folding pathway. Here the discussion of post-translational folding is skipped and only co-translational folding highlighted.

CONCEPTS OF CO-TRANSLATIONAL PROTEIN FOLDING PROCESS AND ITS REGULATION

Preliminary evidence of co-translational folding for at least some proteins appeared around the same time as Anfinsen performed his seminal experiments on ribonuclease (Cowie et al., 1961; Kiho & Rich, 1964). In theory, full-length unfolded polypeptide was energetically unfavorable (Fedorov & Baldwin, 1997) so that the folding process of most proteins should be more or less co-translational. How co-translational folding proceeds is another complicated point. As for the site of co-translational folding, there are two major steps of co-translational folding. Firstly, newly synthesized polypeptide had to travel through a ribosomal exit tunnel of approximately 80 angstrom in length (Fedyukina & Cavagnero, 2011), which is wide enough to accommodate α-helix formation. Indeed, α-helix within exit tunnel has been directly confirmed by FRET (fluorescence resonance energy transfer) (Woolhead et al., 2004). Additional evidence suggested that exit tunnel could entropically stabilize both α-helix (Ziv et al., 2005) and distinct conformations of nascent polypeptide via extensive contacts with ribosomal components (Bhushan et al., 2010). Secondly, other steps of co-translational folding, especially the higher order ones impossible within confined space of exit tunnel, occurred after a partial exit of polypeptide from ribosome. For example, co-translational folding of cystic fibrosis transmembrane conductance regulator was dissected experimentally (Kim et al., 2015). And its α-subdomain compaction was delayed until all related polypeptides migrated into cytosol (Kim et al., 2015).

Consistent with our understandings of co-translation protein folding, many *cis* and *trans* regulators have been implicated. Some discovered *trans* regulators include ribosome-bound chaperones capable of operationally extending exit tunnel and providing additional space for protein folding (Kramer et al., 2009). Also co-translational recognition by signal recognition particle (SRP) induced a translocation of nascent peptide into endoplasmic reticulum with a distinct folding environment (Pechmann et al., 2014). As for *cis* regulators, two distinct and yet probably synergistic mechanisms affect co-translational folding (Pechmann et al., 2014). One mechanism operates by recruiting certain *trans* regulators through specific motifs such as SRP-binding elements (Pechmann et al., 2014) while another by modulating elongation speed (O'Brien et al., 2014, 2012). Two mRNA features have been implicated in modulating elongation speed and thus regulating co-translational protein folding, without altering peptide sequences, i.e. codon optimality (Pechmann et al., 2014; Zhang et al., 2009) and mRNA secondary structures (Faure et al., 2016; Jia et al., 2004; Liu & Liu, 1999; Zhang et al., 1998).

IMPACT OF CODON OPTIMALITY ON PROTEIN FOLDING

It was known that 18/20 amino acids are encoded by two or more synonymous codons. Among them, some are called "optimal" because of either their higher thermodynamic stability after pairing with anticodon or a higher abundance of their cognate tRNAs. The current mechanistic model of translational elongation dictates that codon optimality influences translational efficiency and/or accuracy (Gingold & Pilpel, 2011), i.e., optimal codons are translated faster (see below) and/or with higher fidelity. Unlike the sparse data for mRNA structure, accumulation of sequenced ORFs facilitated codon optimality profiling in a wide array of genes and species since 1970s, resulting in a large body of work investigating codon optimality-dependent modulation of translational elongation rate and its effect on co-translational protein folding. For example, Escherichia coli multidomain protein SufI was examined. Severe perturbation was reported for SufI folding efficiency by excessive tRNA *in vitro* or synonymous substitution into some clusters of non-optimal codons. It was assumed that the clusters of non-optimal codons transiently attenuated translational elongation, temporally separated the translation of segments of peptide chain and actively coordinated co-translational folding. Considering tRNA supply and demand, Pechmann and colleagues (Pechmann & Frydman, 2013) modeled efficiency of translational elongation in 10 closely related yeast species, and found evolutionarily conserved distribution of codon optimality that is associated with secondary structure of translated polypeptides. The authors suggested that mRNA sequences,

and in particular synonymous codon choices, are generally under selection to optimize the co-translational folding of corresponding polypeptides. Altogether, these and other reports (Komar, 2009) have hinted at an evolutionarily conserved link between clusters of non-optimal codons and pauses of translational elongation that facilitates co-translational protein folding, and more importantly, the existence of additional genetic information in an ORF beyond primary protein sequence. Nevertheless, the exact molecular mechanism for such regulatory effect remains elusive, as cluster of non-optimal codons could have been evolved due to selection for sequence features other than the non-optimality of codons.

MESSENGER RNA STRUCTURE AFFECTS TRANLATIONAL ELONGATION

As a critical component of genetic information flow for a certain protein coding gene, messenger RNA extracts coding sequences from genome and applies it as a template for protein synthesis. Nevertheless, not merely a sequence of codons, mRNA has its own complex structures. In particular, its secondary structure of Watson-Crick pairing between nucleotides could regulate translational processes at multiple levels. For example, stable secondary structure at the 5' end of mRNA might suppress translational initiation and thus enhance overall translation efficiency in *E. coli* (Kudla et al., 2009). Excessive stable stem regions at the 3' UTR decreased the accessibility of miRNA response elements and interfered with miRNA-mediated translational repression (Kertesz et al., 2007). While the above examples were limited to either end of mRNA, other functional roles of mRNA secondary structure have been discovered for major coding sequences, such as regulating RNA editing (Nishikura, 2006) and splicing (Shepard & Hertel, 2008). Furthermore, secondary structure of nascent mRNA might lower local mutation rate (Chen et al., 2016). More importantly, specific mRNA structure could interfere with the movement of translating ribosome (Brierley et al., 1991; Chen et al., 2013; Qu et al., 2011; Wen et al., 2008). Given numerous reports (Ciryam et al., 2013; O'Brien et al., 2014, 2012; Wang et al., 2015) connecting translational elongation rate to co-translational folding, it is thus not surprising that theoretical studies have already linked mRNA secondary structure to protein folding.

EARLY EVIDENCE FOR THE REGULATORY ROLE OF mRNA STUCTURE ON PROTEIN FOLDING

The earliest report correlating mRNA structure with the regulation of protein folding appeared in 1993 (Guisez et al., 1993). In the study of Guisez et al., several nascent polypeptide intermediates of coat protein of RNA bacteriophage MS2 were analyzed. And the sizes of nascent polypeptide intermediates were found corresponded to either the positions of rare codons or RNA regions with double-stranded secondary structures, both presumably decrease the velocity of translating ribosomes. It was thus hypothesized that discontinuous translational elongation rate generally facilitates optimal folding of polypeptides. The hypothesized regulated protein folding by mRNA structure was later supported by two additional empirical analyses. On the one hand, the codons of hydrophobic and hydrophilic amino acids tend to respectively located in stem and loop regions of mRNA (Zhang et al., 1998). Given the crucial role of hydrophobic effect on stabilizing protein structure, such observation is suggestive for the information transfer between mRNA and protein structure (Zhang et al., 1998). On the other hand, experimentally determined protein secondary structures were directly compared with computationally predicted mRNA secondary structures (Jia et al., 2004). And α-helices and β-strands within a folded protein tend to be encoded by double-stranded mRNA regions whereas random coils within polypeptide were more likely to be encoded by single-stranded mRNA regions. Although these studies offered preliminary evidence for an intriguing link between mRNA and protein structure, their limitations were also obvious. On the one hand, due to the scarcity of experimentally determined mRNA structure, these studies resorted to computationally predicted mRNA secondary structure with a modest accuracy at best (Lange et al., 2012), let alone higher level structures. On the other hand, the proposed link between mRNA and protein structure was mediated by the capability of mRNA structure in modulating translational elongation speed, whose exact nature was mostly unknown by then. Recent genomic advances have enabled assessments of the above link.

NOVEL GENOMIC DATA QUESTION THE REGULATION OF PROTEIN FOLDING BY CODON OPTIMALITY

The advances of high throughput sequencing techniques have allowed experimental explorations for both translational elongation speed and mRNA structure at the genomic levels (Graveley, 2016; Ingolia et al., 2012). And the resulting datasets have triggered empirical tests of two major hypotheses for *cis*-regulatory signal in mRNA for co-translational folding, i.e. codon optimality and mRNA structure (Qian et al., 2012; Tuller et al., 2011; Yang et al., 2014). Overall, the effect of codon optimality failed to receive consistent supports. The rationales for the regulatory role of mRNA structure in protein co-translational folding and its relationship with codon optimality shall be summarized in the next section.

Essentially as a snapshot for the distribution of ribosomes within transcriptome, ribosome profiling (Ingolia et al., 2009) utilized high-throughput sequencing of segmental mRNA shielded by translating ribosomes from endonuclease digestion, Since translating ribosomes spend more time on stretches of nucleotides with higher coverage in ribosome profiling than other nucleotides in the same gene, such detail ribosomal kinetics allowed revelation/confirmation of several critical features of translational elongation. Firstly, translational elongation rate was not uniform among different mRNAs or along a single mRNA molecule (Ingolia et al., 2009, 2011). Secondly, strong ribosomal pauses lasting over a couple of seconds, >10 times slower than average elongation speed, were widely distributed (Ingolia et al., 2011). Thirdly, at least some variations of elongation speed within gene was obviously non-neutral and had evolved under natural selections (Tuller et

al., 2010). All the above findings were consistent with the model of co-translational protein folding as regulated by translational elongation rate, necessitating the validation of codon optimality and/or mRNA structure as a regulator of elongation rate.

Indeed existing data of ribosome profiling have enabled independent assessments of the role of codon optimality in the control of translational elongation speed. Unexpectedly, several attempts of confirming the slow translational speed of individual non-optimal codons failed to reveal any signal in genomic ribosome profiling data of several species, including yeast (Charneski & Hurst, 2013; Qian et al., 2012), worms (Stadler & Fire, 2011), rodents (Ingolia et al., 2011) and bacteria (Li et al., 2012). These studies explicitly tested the correlation between codon optimality and elongation speed, and found negative results so that other determinants of elongation speed were examined, such as positively-charged amino acids (Charneski & Hurst, 2013), wobble base-pairing (Stadler & Fire, 2011) and anti-Shine-Dalgarno sequence (Li et al., 2012). In one of these studies, observations were explained by balanced synonymous codon usage of transcriptome relative to the abundance of tRNA (Qian et al., 2012). When there was an overall shortage of translation-ready tRNAs, balanced codon usage makes tRNA shortage for all codons similar. It avoided long ribosomal pauses caused by extreme tRNA shortage for a few codons and thus minimizing transcriptome-wide total duration of ribosomal pauses. Further analyses confirmed such a balanced codon usage for multiple eukaryotic transcriptomes. It hinted at adaptive evolution towards balanced codon usage, which presumably provides optimal allocation of translational resources and alleviated ribosomal sequestering due to translational pauses. As experimentally validated, global translational efficiency increased after a heterologous gene with balanced codon usage was transfected into yeast cells, compared to a gene using only optimal or non-optimal codons. The experimental observations were consistent with codon harmonization (Angov et al., 2008), a strategy commonly employed to enhance heterologous protein expression in synthetic biology. More importantly, the model of balanced codon usage indicates that previous experimental results correlating non-optimal codons with halt of translational elongation could be artefactual since most of them involved transfecting a highly expressed heterologous gene into a host cell. A high expression of heterologous gene perturbed the balance between codon usage and tRNA supply. Since the absolute number of cognate tRNA for optimal codon was higher, tRNA shortage was thus proportionally less serious for optimal than non-optimal codons so that there was faster translational elongation for optimal codons in heterologous system. Collectively, the above results cast doubts over the conventional wisdom of faster translation of optimal codons (Charneski & Hurst, 2013; Ingolia et al., 2011; Qian et al., 2012; Yang et al., 2014).

Nevertheless, the results suggesting no correlation between codon optimality and ribosomal velocity are not without their own problems. Most notebly, when cycloheximide was used for stabilizing ribosomes prior to position measurements, elongation re-occurred in the presence of cycloheximide but with dramatically altered codon-specific elongation rates. And the measured positions of ribosomes failed to reflect the temporal durations of ribosomal pausing at each position *in vivo* (Hussmann et al., 2015). Meanwhile, other studies have independently examined the correlation between codon optimality and translational elongation rate, but inconsistent results were obtained (Gardin et al., 2014; Li, et al., 2012; Stadler & Fire, 2011). After analyzing multiple datasets of ribosome profiling, it was found that, regardless of using cycloheximide or not prior to cell lysis, the reproducibility of ribosome profiling was poor at codon resolution since signals at this level were not well-reproduced in experimental replicates (Diament & Tuller, 2016). Previous theoretical and experimental results have confirmed the regulation of co-translational protein folding by clusters of non-optimal codons through modulation of elongation speed. However, the exact molecular mechanism for non-optimal codon cluster stalling ribosomal movement has remained elusive. It left the possibilities of alternative or synergistic regulations other than codon optimality, such that the clusters of non-optimal codons are probably byproducts of other sequence features.

NOVEL GENOMIC DATA SUPPORTING THE REGULATION OF PROTEIN FOLDING BY mRNA STRUCTURE

The development of high-throughput sequencing has enabled multiple methods for examining RNA secondary structure at a genomic level. Early attempts of FragSeq (Underwood et al., 2010), PARS (Kertesz et al., 2010) and SHAPE-seq (Lucks et al., 2011) utilized P1 nuclease, RNase V1 & S1 nuclease and 1-methyl-7-nitroisatoic anhydride respectively, to probe the structures of a large pool of synthetic RNAs or total RNA after extraction from cells, which revealed *in vitro* pairing status of individual nucleotides on RNA molecules. These approaches were followed by the development of mod-seq (Talkish et al., 2014), DMS-seq (Rouskin et al., 2014), Structure-seq (Ding et al., 2014), icSHAPE (Spitale et al., 2015) and SHAPE-Map (Smola et al., 2015), which were capable of detecting *in vivo* RNA secondary structure. More recently, techniques have been developed for detecting pairing partners, including RPL (Ramani et al., 2015), PARIS (Lu et al., 2016), SPLASH (Aw et al., 2016) and LIGR-seq (Sharma et al., 2016). These advanced techniques have cleared the obstacles of genomic RNA structure investigation and enhanced the profiling accuracy of RNA secondary structure (Lange et al., 2012).

Combined with ribosomal profiling data, genomic profiles of RNA secondary structure supported the role of mRNA structure in modulating elongation speed (Tuller et al., 2010, 2011; Yang et al., 2014) (but see Charneski & Hurst, 2013). In particular, comparisons among genes revealed stronger mRNA secondary structure (Zur & Tuller, 2012) and slower translational elongation (Yang et al., 2014) for highly expressed genes, which is more sensitive to protein misfolding (Yang et al., 2010; Zhang & Yang, 2015). Additional comparisons within gene revealed that mRNA pairing status at the entrance of ribosome had the strongest impact upon elongation rate (Yang et al., 2014). This result was consistent with single molecule level

study of individual translating ribosome using optical tweezers, who found that pausing duration of ribosomal translocation was significantly dependent on mRNA secondary structure (Wen et al., 2008). More importantly, the regulatory effect of mRNA secondary structure on ribosome velocity seemed independent of codon optimality (Yang et al., 2014). Thus mRNA secondary structure might serve as a regulator of protein co-translational folding via modulating elongation speed. During translation, mRNA secondary structures were actively unfolded by ribosomes (Rouskin et al., 2014). However, the distance between adjacent ribosomes was approximately 20-35 nm in eukaryotes. And it was translated into 50-90 nt or 17-30 codons, allowing enough time for intervening mRNA to refold given the thousand fold difference between timescale of ribosomal elongation (~0.1s per codon (Ingolia et al., 2009, 2011) and RNA folding kinetics (10^{-5} s for folding a simple hairpin (de Smit & van Duin, 2003)).

More recently, new genomic data were used for directly testing the connection between mRNA structure and protein folding by comparison among genes (Faure et al., 2016). Using protein structures from 2 eukaryotes and 3 prokaryotes, protein compactness was positively correlated with the stability of mRNA structure. Such correlations are more pronounced in ordered parts than disordered parts of protein. Thus it suggested an important role of mRNA secondary structure in modulating protein folding. More importantly, comparison with translational efficiency inferred from ribosome profiling data supported that stable mRNAs were translated slowly to allow more time for compact proteins to fold co-translationally (Faure et al., 2016).

Collectively, new genomic data of ribosome profiling and mRNA secondary structure suggested a mechanistic model (Fig. 1), where the co-translational protein folding is regulated by mRNA secondary structure through its modulation of translational elongation rate. Such a regulation was independent of elongation slowdown due to nonoptimality of synonymous codon usage, whose capacity of regulating co-translational protein folding has remained debated.

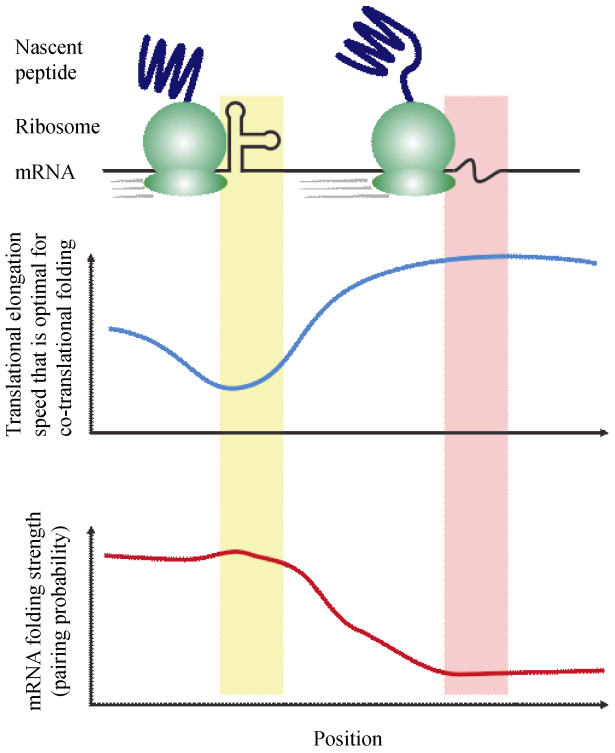

Figure 1 A mechanistic model of co-translational protein folding under the regulation of mRNA secondary structure

Accuracy of co-translational folding is affected by translational elongation speed so that optimal elongation speed varies for different regions within coding sequences. For regions where slow elongation is optimal, mRNA forms stable secondary structure to slow down the movement of ribosomes (highlighted by yellow box). On the contrary, single-stranded mRNA enables faster elongation in regions where higher elongation rate is preferred for accurate co-translational folding (highlighted by pink box).

BIOMEDICAL IMPLICATIONS

In conclusion, elucidating the regulatory role of mRNA secondary structure for protein folding shall expand our understandings of the full contents of genetic information and the molecular mechanisms of its phenotypic expression. Gaining such insights offers broad implications for biological researches. For example, combined with proper bioinformatic algorithm for designing mRNA structure, it is bound to enhance our capability of expressing functionally heterologous proteins in cells. The generality of this regulatory role has raised questions on the neutrality of synonymous variations in coding sequences for molecular evolutionary analyses. The regulatory role of mRNA structure in protein folding has become the only model capable of explaining the stronger mRNA secondary structures in highly expressed genes (Yang et al., 2014; Yang & Zhang, 2015). It offers a fundamental tool of understanding how natural selection concerts the optimality of synonymous codon usage and mRNA secondary structure and subsequently affects the evolution of coding sequences. Co-translational misfolding is a form of phenotypic mutation. The regulatory role of mRNA structure in both protein folding and mutation rate may lead to a quantitative coupling between genotypic and phenotypic mutation rates (Chen et al., 2016). Full biological ramifications of such intriguing coupling between processing and transmission fidelity of genetic information await further explorations.

Detailed modeling of co-translational folding modulated by mRNA structure can help us predict or interpret the phenotypic effects and elucidate the underlying mechanisms of synonymous variations ubiquitous in human genome (The 1000 Genomes Project Consortium, 2010). Implicated in human diseases (Kimchi-Sarfaty et al., 2007), it is considered as frequent driver mutations in human cancer (Supek et al., 2014). Altered mRNA structure might result in a dysregulation of co-translational protein folding, leading to protein misfolding and aggregation that is disproportionately involved in neurodegenerative diseases (Soto, 2003). Understanding the

subtle roles of mRNA structure in protein misfolding and aggregation shall reveal new therapeutic targets for neurodegenerative diseases.

ACKNOWLEDGEMENTS

Great appreciations were extended to Yong-Gang Yao, Gong-Wang Yu, Heng Zhang and two anonymous reviewers for valuable comments.

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Expression of pIgR in the tracheal mucosa of SHIV/SIV-infected rhesus macaques

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ABSTRACT

Polymeric immunoglobulin receptors (pIgR) are key participants in the formation and secretion of secretory IgA (S-IgA), which is critical for the prevention of microbial infection and colonization in the respiratory system. Although increased respiratory colonization and infections are common in HIV/AIDS, little is known about the expression of pIgR in the airway mucosa of these patients. To address this, the expression levels of pIgR in the tracheal mucosa and lungs of SHIV/SIV-infected rhesus macaques were examined by real-time RT-PCR and confocal microscopy. We found that the levels of both *PIGR* mRNA and pIgR immunoreactivity were lower in the tracheal mucosa of SHIV/SIV-infected rhesus macaques than that in non-infected rhesus macaques, and the difference in pIgR immunoreactivity was statistically significant. IL-17A, which enhances pIgR expression, was also changed in the same direction as that of pIgR. In contrast to changes in the tracheal mucosa, pIgR and IL-17A levels were higher in the lungs of infected rhesus macaques. These results indicated abnormal pIgR expression in SHIV/SIV, and by extension HIV infections, which might partially result from IL-17A alterations and might contribute to the increased microbial colonization and infection related to pulmonary complications in HIV/AIDS.

Keywords: Tracheal mucosa; Lungs; pIgR; SHIV/SIV infection; IL-17A

INTRODUCTION

The respiratory system is continuously exposed to foreign antigens from either airborne or commensal microbes. Due to vulnerability of the physical epithelial barrier of the respiratory system, most pathogens are stopped from entering the body by the mucosal immune system. A key component of the airway mucosal immune system that prevents microbial infections and colonization is secretory IgA (S-IgA), which is composed of dimeric IgA produced in the lamina propria and extracellular part of the polymeric immunoglobulin receptors (pIgR), also known as the secretory component (SC) expressed by mucosal epithelial cells (Johansen & Kaetzel, 2011).

Newly synthesized pIgR is localized to the basolateral surfaces of mucosal epithelial cells, where it binds to dimeric IgA (dIgA) and mediates transcytosis of IgA to the apical surface of the epithelial cells (Johansen et al., 1999). The SC can be released to the mucosal surface alone (in the absence of IgA) or together with dIgA as S-IgA. In addition, SC bound to dIgA can elongate the life of S-IgA and enhance its immune exclusion ability. It can also stop microbial invasion. Mice deficient in pIgR expression are reportedly unable to control infections of the airway by some bacteria, which could drive progressive chronic obstructive pulmonary disease (COPD) phenotype in these mice (Richmond et al., 2016).

Pulmonary complications are common and major causes of morbidity and mortality in HIV-infected individuals, even in the presence of highly active antiretroviral therapy (ART) (Grubb et al., 2006; Murray, 1996). Increased pulmonary infections and microbial colonization are common in HIV/AIDS patients (Zar, 2008). Whether and how the S-IgA/pIgR system is involved in these alterations is not well addressed. Rhesus macaques are important in HIV/AIDS studies. In previous research, we found that pIgR expression was altered in the gut mucosa of SHIV/SIV-infected rhesus macaques (Wang & Yang, 2016). To determine whether pIgR is involved in the respiratory pathology of HIV/AIDS, we examined the expression of pIgR in the tracheal mucosa of SHIV/SIV-infected rhesus macaques.

MATERIALS AND METHODS

Tissues

Tissue samples from the tracheas and lungs were collected from five normal and five SHIV/SIV-infected rhesus macaques

Received: 11 October 2016; Accepted: 10 November 2016

Foundation items: The study was supported by the Beijing Natural Science Foundation (7162136)

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DOI:10.13918/j.issn.2095-8137.2017.007

(*Macaca mulatta*), as reported previously (Wang & Yang, 2016). The sites from which samples were collected were chosen randomly. Tissue samples for RNA isolation were frozen on dry ice immediately after collection and preserved in a freezer at -80 °C before use. Tissue samples for confocal microscopy were fixed in 4% paraformaldehyde immediately after collection, then washed and protected with 30% sucrose, and finally embedded in OCT and preserved in a freezer at -80 °C. All study animals were treated humanely per the state and local regulations on the care and use of experimental animals.

Real-time RT-PCR

Quantification of pIgR and IL-17A mRNA levels was conducted by TaqMan® probe real-time RT-PCR, as performed previously (Wang & Yang, 2016; Zhang et al., 2014). Briefly, RNA was isolated using a RNAprep Pure Tissue Kit (Tiangen Biotech, China) per the manufacturer's protocols. Real-time PCR mixtures were established with a One Step PrimeScript® RT-PCR Kit (Takara, Japan) and primers and probes for pIgR and IL-17A (Wang & Yang, 2016; Zhang et al., 2014). PCR was performed on a 7500 Real-Time PCR System with 7500 System SDS software version 1.4 (ABI, USA). GAPDH mRNA levels in all samples were used as internal controls.

Confocal microscopy

Tissue sections were cut with a cryostat LEICA CM 1850 (Leica Inc., Germany) to a thickness of 20 microns. After removing the OCT with PBS supplemented with 0.1% Triton X-100 and FSG, the slides were washed with PBST and blocked with 10% normal goat serum for 1 h before incubation in polyclonal antibody against human pIgR (rabbit anti-PIGR, 4 μg/mL, Abcam, USA) at 4 °C overnight. Sections were incubated in secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit IgG, 2 μg/mL, Invitrogen, USA) for 1 h after washing off the extra primary antibody. Slides were washed and mounted with anti-fade mounting medium and observed with an Olympus FV1000D-ST confocal microscope (Olympus, Japan). Images (1024×1024) were acquired and morphometric measurements were obtained with Image-Pro Plus software version 6.0 (Media Cybernetics, Silver Springs, MD, USA).

Statistics

All quantitative parameters were expressed as mean±*SD*. Non-parametric Mann-Whitney *U* test was used to compare the means of parameters between normal and infected rhesus macaques. Spearman test was used to calculate the correlations between pIgR mRNA and IL-17A mRNA levels. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Localization of pIgR immunoreactivity in the tracheal mucosa of rhesus macaques

To detect the expression of pIgR in the tracheal mucosa of rhesus macaques, pIgR immunoreactive cells were examined with confocal microscopy. As shown in Figure 1, pIgR immunoreactivity was detected with a polyclonal antibody against human pIgR. In the epithelium, immunoreactivity to pIgR was localized to both the apical and basolateral surfaces of the epithelial cells. It was also localized in the cytoplasm of the basal part (under the nucleus) of the epithelial cells. After SHIV/SIV infection, pIgR immunoreactivity was lower in the tracheal mucosa of rhesus macaques.

Expression of pIgR decreased in the tracheal epithelium of SHIV/SIV-infected rhesus macaques

To determine changes in pIgR expression after SHIV/SIV infection, levels of pIgR immunoreactivity were quantitatively examined with Image-Pro Plus software and pIgR mRNA levels were examined by real-time PCR. As shown in Figure 2, levels of pIgR immunoreactivity were 1.65 times higher in the tracheal epithelium of normal rhesus macaques than that in SHIV/SIV-infected rhesus macaques (Figure 2A), with statistical significance (Mann-Whitney *U* test, *P*=0.007 9). The transcription levels of pIgR genes in the tracheal mucosa of normal rhesus macaques were 1.57 times higher than that in infected rhesus macaques, although the difference was not statistically significant (Mann-Whitney *U* test, *P*=0.254 4). Therefore, both the transcription and protein levels of pIgR were about 1.6 times higher in normal than in infected rhesus macaques.

IL-17A is a regulator of pIgR expression and is decreased in HIV and SIV infection. We examined the transcription levels of IL-17A in the tracheal mucosa of normal and infected rhesus macaques. IL-17A mRNA levels in the tracheal mucosa of normal rhesus macaques were 1.8 times higher than that in SHIV/SIV-infected rhesus macaques (Figure 2C), although the difference did not reach statistical significance (Mann-Whitney *U* test, *P*=0.5476). Positive correlation was observed between pIgR and IL-17A mRNA levels in the tracheal mucosa of normal rhesus macaques (Figure 2D), though this trend was not found in SHIV/SIV-infected rhesus macaques.

Expression of pIgR in the lungs of SHIV/SIV-infected rhesus macaques

To determine whether the lungs of SHIV/SIV-infected rhesus macaques were similarly affected, the expressions of pIgR mRNA and IL-17A mRNA in the lungs of normal and infected rhesus macaques were examined. The mRNA levels of pIgR and IL-17A were 50 and 32 times higher, respectively, in the tracheal mucosa than in the lungs. As shown in Figure 3, pIgR and IL-17A mRNA were both detected in the lungs of normal and infected rhesus macaques. In contrast to the changes observed in the tracheal mucosa, the levels of pIgR and IL-17A mRNA were 3 and 1.2 times higher, respectively, in infected rhesus macaques than in normal rhesus macaques, although the differences were not statistically significant (Mann-Whitney *U* test, *P*=0.4396 and 0.7857, respectively). Therefore, the expressions of pIgR and IL-17A were higher in the tracheal mucosa than in the lungs, and were not reduced in the lungs of SHIV/SIV-infected rhesus macaques.

DISCUSSION

In the present study, we observed reduced expression of pIgR

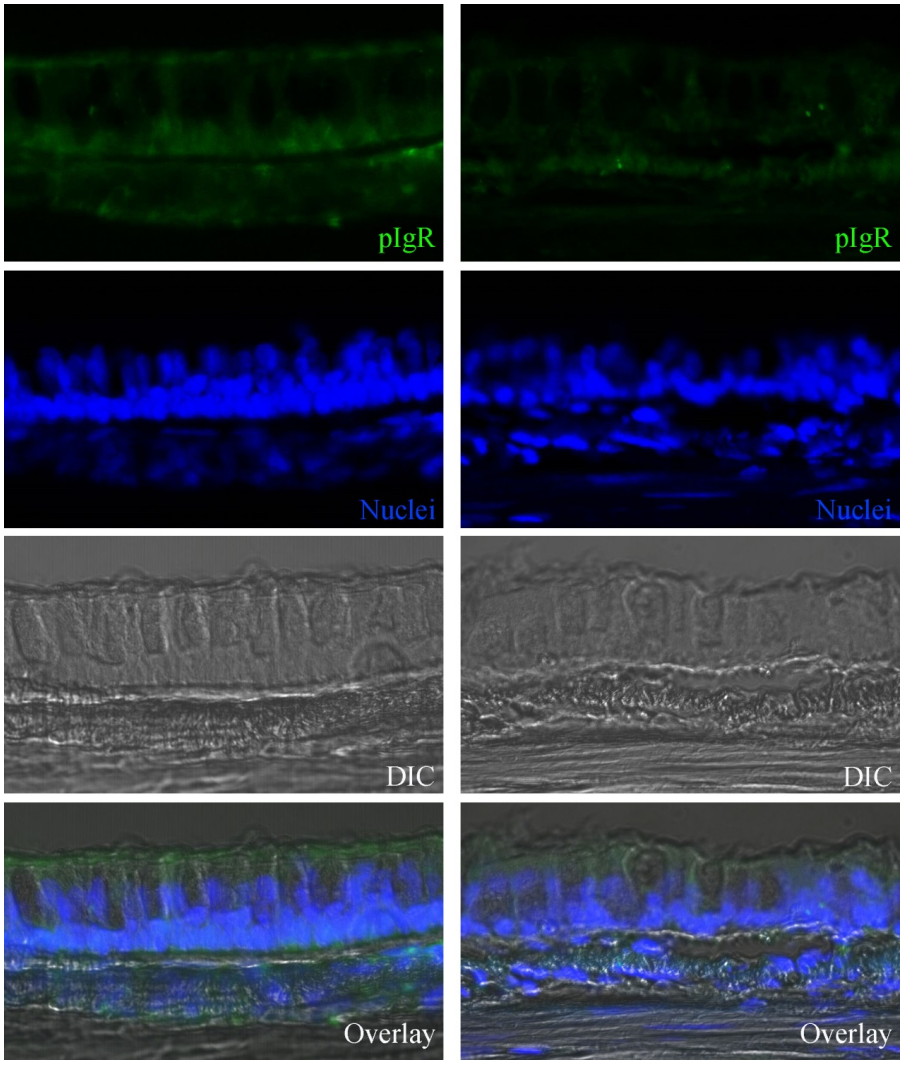

Figure 1 Distribution of pIgR immunoreactivity in the epithelium of tracheal mucosa from rhesus macaques

Immunoreactivities to pIgR (green) in the pseudo-stratified columnar epithelium of tracheal mucosa from normal (left column) and SHIV/SIV-infected (right column) rhesus macaques are shown. Nuclei (blue, stained with DAPI) and DIC images indicate that histological alterations also occurred in the tracheal mucosa of SHIV/SIV-infected rhesus macaques. Original magnification, ×800.

in the tracheal mucosa of SHIV/SIV-infected rhesus macaques. Both the protein levels and mRNA levels of pIgR were decreased to almost the same degree, although the decrease in protein levels was statistically significant, whereas that of mRNA was not. It is possible that the effects of SHIV/SIV infection on pIgR expression were at the gene transcription level. In consistent with these results, previous research showed that pIgR mRNA levels were significantly reduced in the intestinal mucosa of SHIV/SIV-infected rhesus macaques (Wang & Yang, 2016). Downregulation of pIgR in airway mucosa has also been documented in other airway diseases (Gohy et al., 2014; Hupin et al., 2013). Since the pathology between SIV and HIV infection is similar, pIgR expression in the airway mucosa of HIV-infected patients could also be significantly affected.

The mechanism of decreased pIgR expression in SHIV/SIV infection has not been addressed. There are many potential factors that could affect pIgR expressiopn, among which IL-17A can significantly regulate pIgR expression (Jaffar et al., 2009). In the present study, a decrease in IL-17A expression in the tracheal mucosa of infected rhesus macaques was observed, suggesting a role of IL-17A in the downregulation of pIgR expression in the context of SHIV/SIV infection. The non-significant difference might be due to the large individual variability and small sample size. Significant correlation between pIgR and IL-17A mRNA has been observed in the intestinal mucosa of these animals and a significant decrease in IL-17A mRNA has also been observed in the intestinal mucosa (Wang & Yang, 2016; Zhang et al., 2014). Further studies are warranted to reveal the mechanism underlying the decrease of pIgR expression in HIV/AIDS.

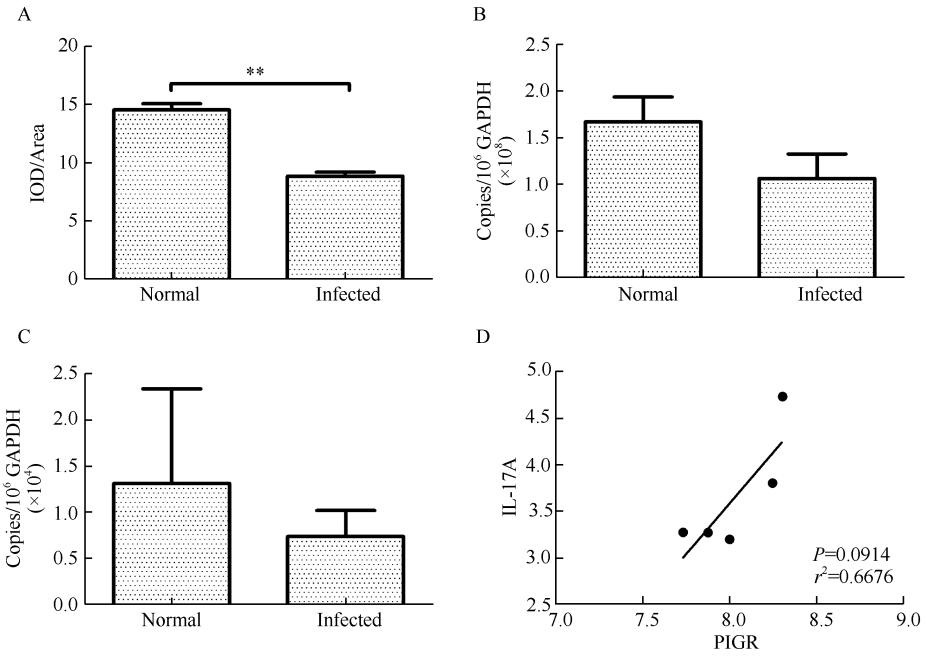

Figure 2 Expression levels of pIgR and IL-17A in the tracheal mucosa of rhesus macaques

Levels of pIgR immunoreactivity (A), pIgR mRNA (B) and IL-17A mRNA (C) in the tracheal mucosa of normal and infected rhesus macaques are shown. mRNA levels of IL-17A and pIgR are positively correlated (D).

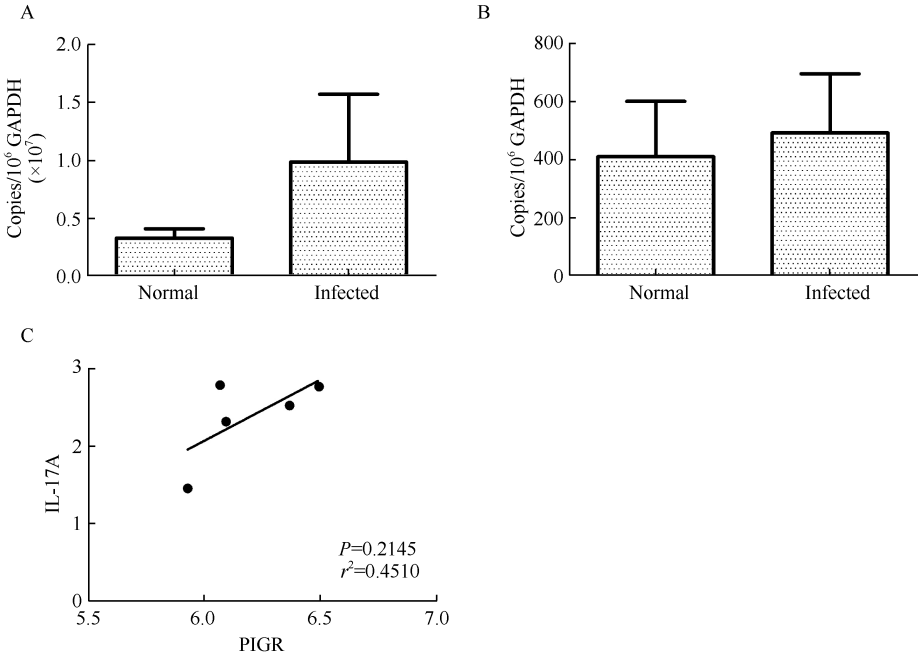

Figure 3 Expression of pIgR and IL-17A in the lungs of rhesus macaques

mRNA levels of pIgR (A) and IL-17A (B) in the lungs of normal and infected rhesus macaques are shown. Correlation between transcription levels of pIgR and IL-17A in normal rhesus macaques is also shown (C).

The consequence of reduced pIgR expression in the tracheal mucosa of SHIV/SIV-infected rhesus macaques is unknown. Nevertheless, these data indicate impaired immune exclusion of potential pathogenic and commensal microbes in the respiratory system. In line with this, increased airway microbes and pulmonary infections have been documented in HIV/SIV infections (Nimmo et al., 2015; Twigg et al., 2016). Since elevated microbes can drive the COPD-like phenotype in pIgR

deficient mice (Richmond et al., 2016) and downregulation of pIgR is observed in COPD patients (Gohy et al., 2014), reduced pIgR expression could be an underlying mechanism of the increased incidence of COPD in HIV/AIDS patients (Morris et al., 2011). COPD is the cause of death in a significant proportion of the HIV/AIDS population. ART treatment does not decrease the incidence of COPD, but is an independent predictor of increased airway obstruction (Gingo et al., 2010). Decreased expression of pIgR might also be involved in other pathological processes of HIV/AIDS, such as lung cancer (Ocak et al., 2012). Therefore, abnormal expression of pIgR should be taken into consideration in novel therapies for pulmonary complications such as COPD.

In summary, for the first time, reduced pIgR expression was observed in the tracheal mucosa of SHIV/SIV-infected rhesus macaques, which might be linked to IL-17A reduction in the tracheal mucosa. The reduced expression of pIgR might be the underlying mechanism of increased pulmonary microbiota and infections in HIV/AIDS. Rhesus macaques are a suitable model for future dissection of the mechanisms underlying respiratory complications in HIV/AIDS.

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Nest survival rate of Reeves's pheasant (*Syrmaticus reevesii*) based on artificial nest experiments

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ABSTRACT

To explore the nest survival rate of Reeves's pheasant (*Syrmaticus reevesii*) and the nest-site factors that affect it, we conducted artificial nest experiments with reference to natural nests at Dongzhai National Nature Reserve (DNNR), Henan Province and Pingjingguan, Hubei Province from April to June 2014 simulating the situation in its early and later breeding season. We also determined distance characteristics of the nest sites by ArcGIS 10.0. Nest survival models were constructed in Program MARK for data analysis. Results indicated that in the early breeding season, the apparent survival rate (ASR) in DNNR (52.4%) was significantly greater than that in Pingjingguan (13.5%), and the ASR in the later breeding season in DNNR (26.7%) was not indistinctively correlated with Pingjingguan (3.2%). The daily survival rate (DSR) in the later breeding season was 93.8% in DNNR and 92.0% in Pingjingguan, respectively. The DSRs were both negatively correlated with nest distance to forest edges and settlements. The DSR in Pingjingguan was positively correlated with nest distance to paths and negatively correlated with nest distance to water sources. However, the DSR in DNNR was negatively correlated with nest distance to paths but positively correlated with nest distance to water sources.

Keywords: Reeves's pheasant; *Syrmaticus reevesii*; Nest survival rate; Artificial nest experiments.

INTRODUCTION

Reeves's pheasant (*Syrmaticus reevesii*) is a rare and endangered endemic species in China. It is listed as Grade II wildlife under national protection (State Council, 1988) and as a vulnerable species by the International Union for the Conservation of Nature and Natural Resources (IUCN, 2015). Due to habitat destruction (Wu & Xu, 1987), poaching (Xu et al., 1996) and predation pressure from natural enemies (Xu et al., 1996), the Reeves's pheasant population is decreasing at a rate of 20% every ten years (Zhou et al., 2015). The threats to Reeves's pheasant survival are considered more severe than those of many Grade I protected species in China (Zhang et al., 2003; Zhou et al., 2015). The Reeves's pheasant is ground nesting and highly vigilant, which makes it difficult to carry out field research and nest tracking (Lei & Lu, 2006; Zhang et al., 2004). Direct observation of incubating females can induce nest abandonment and breeding failure (Collar et al., 1994), and thus only limited information is known in regards to their nest survival rate.

Nest building, egg laying and egg hatching are not only critical stages in bird breeding seasons, but are also relatively precarious and easily affected by adverse environmental factors (Welty, 1962). Nest success rate is one of the most important factors determining fertility and has significant effects on bird population quantities (Beale & Monaghan, 2004). Accurate estimates of nest success rate and relevant influencing factors are important to understand the quantitative dynamics of bird populations and meaningful to enact suitable protective policies for endangered species (Lindell et al., 2011).

Artificial nest experiments using quail (*Coturnix japonica*), chicken, or artificial plaster eggs to similar to natural nests have been used to investigate predation risks of nestling (Reitsma et al., 1990; Sun et al., 2011). In addition, because it is easy to manipulate and permitting to control the experimental conditions, artificial nest experiments have also been popular in the studies on nest survival rate (Major & Kendal, 1996; Martin, 1987; Sieving, 1992; Sun et al., 2011; Willebrand & Marcström, 1988), and are a valuable reference for natural nesting survival studies (Wilson et al., 1998). Wang et al. (2016) used artificial nest experiments to study predation of the Reeves's pheasant. Results indicated that, to a certain extent, artificial nest experiments can reflect the fates of natural nests. Referencing

Received: 28 September 2016; Accepted: 10 November 2016

Foundation items: This study is supported by the Forestry Commonweal Program (201404422) and the National Natural Science Foundation of China (31172115)

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DOI:10.13918/j.issn.2095-8137.2017.008

the methods used in Wang et al. (2016), we conducted artificial nest experiments on Reeves's pheasant at Dongzhai National Nature Reserve (DNNR), Henan Province and Pingjingguan, Suizhou City, Hubei Province from April to June 2014. Experiments were based on the characteristics of natural nests collected from field surveys in recent years, with chicken eggs used as mock eggs. We aimed to provide evidence on the nest survival rates of Reeves's pheasant, and the effects of distances from nest sites to various habitat parameters (e.g., forest edges, paths, water sources, settlements) on survival rates.

MATERIALS AND METHODS

Study area

The experiments were carried out at Baiyun Protection Station, DNNR, Henan Province (N31°28′-32°09′, E114°18′-114°30′) and Pingjingguan, Suizhou City, Hubei Province (N31°51′-31°52′, E113°54′-113°55′). The two areas are in the south foothills of Dabieshan Mountain, less than 40 km apart and both approximately 400 hm^2 in area. The geological conditions, precipitation, temperature and phenology are similar (details in Wang et al., 2016).

Locating natural nests

Natural Reeves's pheasant nests in Pingjingguan were located in two ways: (1) Fouteen nests were found by radio telemetry during field surveys from 2011 to 2013, seven nests were found during this study from ten captured females caught in March 2013; and, (2) Four nests were found by interviewing local residents undertaking farming and firewood, herb collection in the mountains. The natural nests included in DNNR were seven nests found by other research teams during 2011 to 2014.

Information on the structures and sites of the fourteen natural nests obtained from Pingjingguan during 2011 to 2013 were used as references for establishing artificial nests. The twenty-five natural nests from Pingjingguan and seven from DNNR during 2011 to 2014 were included in evaluating and comparing the survival rates between natural and artificial nests.

Artificial nest experiments

The locations of the artificial nests were chosen within the areas in which Reeves's pheasants were found by radio telemetry during previous research (Sun et al., 2003; Xu et al., 2007; Bai, 2013). The selection of the artificial nests was determined based on the habitat characteristics of fourteen natural nests found during field surveys from 2011 to 2013. The artificial nests were constructed by mimicking the structures of natural nests (Wang et al., 2016). The artificial nests were disk-like, padded with withered and yellow *Castanea mollissima* leaves, *Pinus tabuliformis* pine needles or straw, and covered with 1-2 down feathers collected from the field. Four chicken eggs of similar size and color to that of Reeves's pheasant were placed in each artificial nest.

Two rounds of artificial nest experiments were conducted. The first round was carried out from late April. When choosing locations by ArcGIS 10.0(Esri Inc. <*http: //www.esri.com/*>), seventy random locations were picked in the two study areas. Except for the locations with rocks, roads, nudations, and ponds, one artificial nest locus was picked within a 100 m^2 range around each of the remaining locations. A total of 46 and 42 artificial nests were placed at Pingjingguan and DNNR, respectively. Using the same method and procedure in the second round of experiments (May 2014), fifty random locations were determined, and a total of 31 and 30 artificial nests were placed at Pingjingguan and DNNR, respectively. As per the descriptions of the four stages of breeding season in Reeves's pheasant (Sun et al., 2003), our two experiments simulated nest survival situations in the early and later stages, respectively.

In natural environments, the hatching period of Reeves's pheasant is 26-27 d (Zhang et al., 2004). In this study, the period of the artificial nest experiments was set at 30 d. Due to adverse weather conditions during the first experiment, the nests were checked three times on day 10, day 15 and day 30. During the second experiment, the nests were checked every five days The inspecting time was defined as interval of checked and setting up day (Dinsmore et al., 2002).

To reduce researcher influence on the experiments, human disturbance was minimized during nest checking, e.g., leaving few footprints around the artificial nest and avoiding behaviors that might affect nest, such as touching the eggs (Driscoll et al., 2005). In the wild, if a nest is disturbed or eggs in the nest are preyed, the female Reeves's pheasant abandon the nests (Johnsgard, 1999). If eggs inside the artificial nest were destroyed, removed or disappeared, the eggs were not replaced (Nour et al., 1993) and the nest was defined as failed (Noske et al., 2008). Otherwise, the nest was considered one survival nest. Moreover, because Reeves's pheasant is precocial, one success nest was defined if it was a survival nest at the last inspecting time.

Distance parameters of nest locations

The GPS locations of both natural and artificial nests were analyzed by ArcGIS 10.0. By referencing the definitions of habitat parameters by Xu et al. (2006) and Wang et al. (2016), the degree of slope (Sld), distance to water resources (Dwt), paths (Dho), forest edges (Dfb) and settlements (Dro) were determined. The degree of slope was calculated by Slope tool and the minimum distance parameters were obtained by Euclidean Distance tool.

Data analysis

To compare our findings with previous reports, the apparent survival rate, i.e. ASR was defined in accordance with the definitions of Driscoll et al. (2005):

$$ASR=n_s/(n_s+n_f) \quad (1)$$

Where, ASR is the apparent survival rate, n_s represents the number of successful nests, and n_f represents the number of failed nests.

χ^2-tests were used to compare the differences in ASRs between artificial and natural nests, as well as the differences in ASRs between the two study areas and two different study periods. The results were examined by SPSS 22.0 and plotting was conducted by Sigma Plot 12.1.

Daily survival rates, i.e. DSRs were calculated by Program MARK (V8.0). Due to the irregular checking intervals in the first

experiment, only data from the second experiment were included in calculating the DSR. In the calculation, five basic variables were used for the nest survival rate obtained by the module Nest Survival within MARK program: (1) setting up day of artificial nests (all in "1"); (2) last survival day of nest; (3) the day of nest fate (failure or success) determined; (4) the fate of nest (failure or success); and (5) quantity of nests with same fate. None of the variables went through standardization. Selecting sin option in Link Function to construct the stable model of the DSR of the artificial nests in the later breeding season, and the DSRs of the two study areas in the later breeding season were calculated.

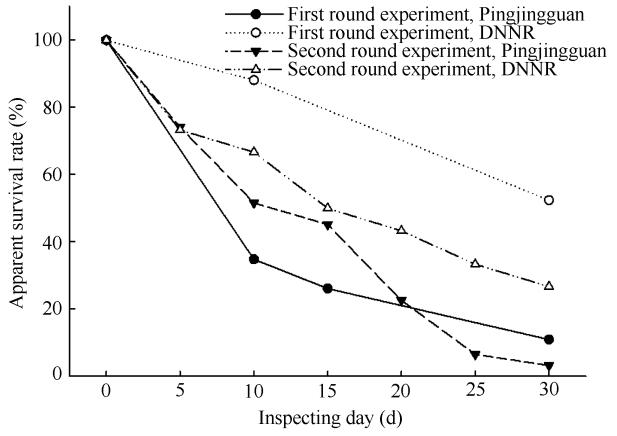
To clarify if degree of slope and distance parameters of nest location to various habitat factors affected DSR, five variables, Sld, Dho, Dfb, Dwt and Dro, were added into the model Nest Survival. By combining the five previously described basic variables, a model regarding the correlations among DSR, degree of slope and distance parameters was constructed. The model average estimated value ($\theta \pm SE$) of each variable and 95% confidence interval, i.e. 95% CI were obtained. The plus-minus estimated value excluding "0" indicated that this variable had positive and negative effects on model construction.

In the results, data of successional variables were presented as mean±*SE*, where, mean is the arithmetical mean and *SE* is the standard deviation. A *P*-value of <0.05 represented significant differences.

RESULTS

Apparent survival rates between artificial and natural nests

The ASRs of the artificial nests in the two study areas decreased with time (Figure 1). At the end of the experiments, the ASRs of artificial nests in Pingjingguan and DNNR were 11.6% (*n*=9) and 41.7% (*n*=30), respectively; that of the twenty-five natural nests in Pingjingguan was 20.0%, and that of the seven natural nests in DNNR was 28.5%. The χ^2-test results showed no significant differences in the ASRs between artificial and natural nests during the two experiments (Pingjingguan: χ^2=0.67, *df*=1, *P*=0.41; DNNR: χ^2=0.20, *df*=1, *P*=0.65).



Figures 1 Apparent survival rates over the two experimental periods in the two study areas

Apparent survival rates of artificial nests at the two study areas in different time periods

At the end of the early breeding season, the ASR of the artificial nests in Pingjingguan was 13.5% (*n*=6), significantly lower than that in DNNR (52.4%, *n*=22) (χ^2=7.65, *df*=1, *P*<0.01). At the end of the later breeding season, the ASR of artificial nests in Pingjingguan was 3.2% (*n*=1), also lower than that of DNNR (26.7%, *n*=8), though the difference was not significant (χ^2=3.81, *df*=1, *P*>0.05). In the two study areas, the ASRs of artificial nests during later breeding season were both lower than that during the early breeding season. However, significant differences were only found in DNNR (Pingjingguan: χ^2=4.75, *df*=1, *P*>0.05; DNNR: χ^2=5.12, *df*=1, *P*<0.05).

Daily survival rate during the later breeding season and its variations with distance and degree of slope

In the later breeding season, the stable model results showed that the DSRs in DNNR and Pingjingguan were 93.8% (*n*=30, CI_{95}=90.8%-95.9%) and 92.0% (*n*=31. CI_{95}= 88.7%-94.3%), respectively.

The distance parameter analyses of model Nest Survival showed that in the later breeding season, DSRs of artificial nests in the two study areas were negatively correlated with distances to forest edges and settlements (Figure 2). In DNNR, the DSR decreased considerably with increasing distance from nest location to forest edges. Moreover, different correlation patterns of DSRs with distance to paths and water resources were found in DNNR and Pingjingguan (Figure 2). In Pingjingguan, the DSR was positively correlated with distance to paths, but negatively correlated with distance to water resources. However, these parameters effects showed the opposite pattern in DNNR.

DISCUSSION

Reeves's pheasants are highly vigilant birds, which makes it difficult to track their nests in the field and carry out monitoring (Lei & Lu, 2006; Zhang et al., 2004). Artificial nest experiments can decrease human disturbance to hatching in the field and provide sufficient samples for research purposes (Major & Kendal, 1996; Sieving, 1992; Sun et al., 2011; Wang et al., 2016; Wilson et al., 1998). In this study, the ASRs of artificial nests during the two experiments were comparable with that of natural nests, indicating their ability to mimic, to some extent, the survival situation of Reeves's pheasant nests in the field.

In the two artificial nest experiments, the ASRs during the later breeding season were both lower than those during the early breeding season. This phenomenon that nest survival rates vary or decrease with time has been reported in previous research (Daan et al., 1990). Our results also support this finding, which could relate to the increase in predation pressure and human disturbance with time (Hatchwell, 1991; Becker & Zhang, 2011).

In the two study areas, during the artificial nest experiments in late May (the later breeding season), the number of raptors, such as *Butastur indicus* and *Accipiter soloensis*, increased, and as they also began to breed, predation pressure to the

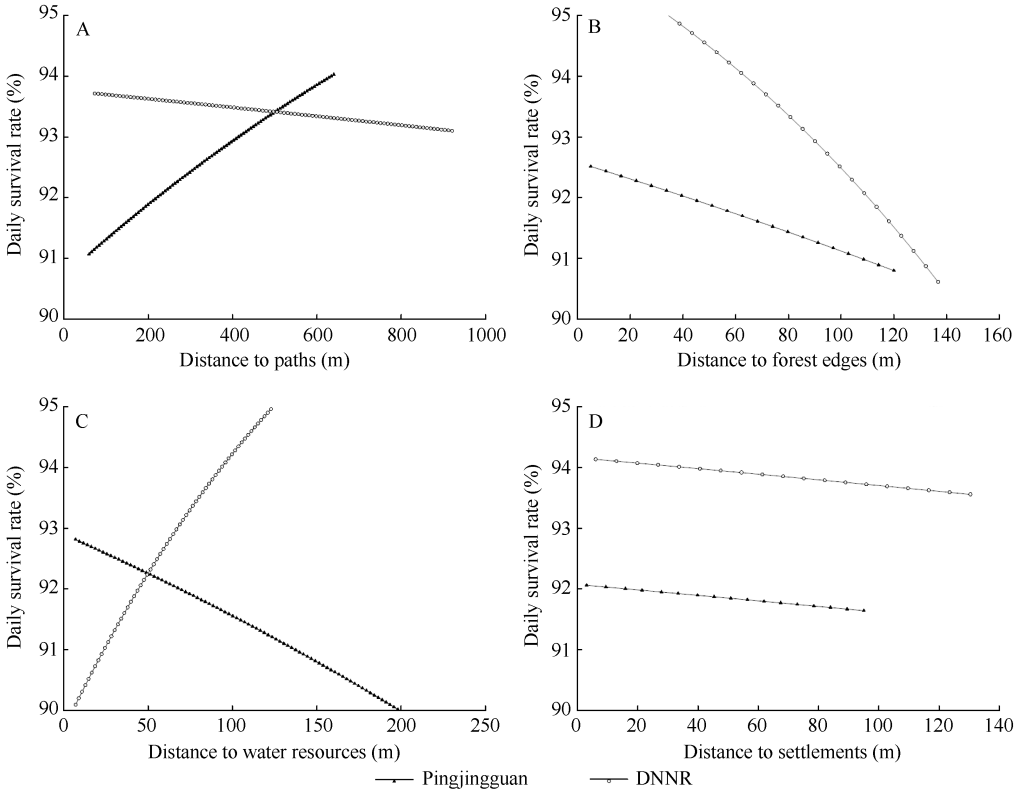


Figure 2 Daily survival rates of artificial nests in the later breeding season with different nest distances to habitat parameters

Reeves's pheasant increased as well. Therefore, predation threats to artificial nests were more severe than that during the early breeding season. According to the local DNNR survey conducted by Ma et al. (2012), because the later breeding season of Reeves's pheasant overlaps with the peak period of busy farming, e.g., pasturing, chestnut weeding, human disturbance in the later breeding season was more intense than that in the early breeding season. In Ma's survey to the community, 35.3% of interviewees claimed to collect Reeves's pheasant eggs from April to June. Sun et al. (2011) stated that repetitive artificial nest experiments in one breeding season could allow predators to adapt to human traces, with increases in predation risks in later experiments.

In this study, in the later breeding season, the DSRs in the two study areas both decreased with increasing distance from nests to the forest edges. The edge effect hypothesis indicates that the edging areas of habitats usually have more enriched vegetation resources and more complicated environments than central areas, so predation pressure at edging areas is higher (Ewers & Didham, 2007; Fahrig, 2003). Our results are discordant with the edge effect hypothesis. It is possible that because human activities are more intense in edging areas, predators, especially those that mammalian predators, are forced into the less disturbed central areas. Therefore, predation pressures in central areas are higher than that in edging areas. Moreover, because it is more common for a nest to be destroyed by a predator than by a human, the effect of predation pressure on nest survival is higher than that of human disturbance. Wang et al. (2016) found that in the later breeding season, the major predation pressure came from predators preying on animals, which supports our assumption to a certain extent. Moreover, the negative correlation between DSR and nest distance to settlements in both study areas also suggests that human disturbance relieved potential predation pressures of artificial nests.

We also found that in the later breeding season, with increasing distance from the nests to paths, the DSR of artificial nests in Pingjingguan increased, whereas that in DNNR showed the opposite trend. The possible reasons for this are that the paths in Pingjingguan are also the main roads used by residents entering the mountain area; therefore, compared with DNNR, the habitats near paths in Pingjingguan have smaller vegetation coverage, fewer herbaceous plants, and higher human disturbance. Therefore, the nests closer to the paths were under higher predation pressure and human disturbance. Moreover, the distance of nests to water resources also had different effects on DSRs in the two study areas. In DNNR, the DSR increased with increasing distance to water resources. The reason might be that predators are more active near water resources, and thus threats from natural enemies decreased with distance to water resources and the artificial nests closer to water resources were under higher predation pressure. However, the situation in Pingjingguan exhibited the opposite pattern, and it was assumed that because water resources, e.g.,

river, penstock, were in areas with more frequent farming activities, disturbance from livestock and herdsman suppressed activities of predators nearby.

In summary, artificial nest experiments were conducted to mimic the survival rates of Reeves's pheasant's nests. Results showed low nest survival rates and high impacts of nest location. Our findings indicate that although data obtained from natural nests allow for more persuasive assumptions (Paton, 1994), to vigilant and endangered species, such as Reeves's pheasant, using artificial nests to mimic natural nests is a useful method in research.

ACKNOWLEDGEMENTS

We thank Qin-Yun Wang, Peng Zhao, and Jun-Qin Hua for help in collecting data in the fields; residents Yuan-Jun Zhang and Jin-Dong Chen for their help; and staff from the Administration Department of Dongzhai National Nature Reserve and Pingjingguan village committee for supporting this study.

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Sponsored by Kunming Institute of Zoology, Chinese Academy of Sciences; China Zoological Society©

Supervised by Chinese Academy of Sciences

Published by Science Press (16 Donghuangchenggen Beijie, Beijing 100717, China)

Printed by Kunming Xiaosong Plate Making & Printing Co, Ltd

Domestic distribution by Yunnan Post and all local post offices in China

International distribution by China International Book Trading Corporation (Guoji Shudian) P.O.BOX 399, Beijing 100044, China

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Domestic Postal Issue No.: 64-20

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ISSN 2095-8137